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*BEHAVIOR OF NONINFECTIOUS SV 40 VIRAL GENOME IN HAMSTER TUMOR CELLS: INDUCTION OF SYNTHESIS OF INFECTIOUS VIRUS**

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Presented before the Academy, April 22, 1963

Basic studies of the behavior of the viral genome in cells experimentally rendered malignant by virus action are not only of general biologic interest but may also provide new approaches to the search for etiologically significant dormant viruses in human cancers. In spontaneously occurring virus cancers of lower animals, in which RNA viruses appear to be implicated, infectious virus is produced by the malignant cells, and the virus etiology of these tumors was in fact readily established by virtue of this property. However, studies on the experimental cancers produced by the DNA polyoma virus of mice failed to reveal persistence of the viral genome in serially transplanted tumors that have been freed of the original virus.[1] Thus, it appeared possible that DNA viruses might completely disappear from cells they have rendered malignant. Recently, we[2] reported studies which indicated that, in a serially transplanted hamster fibrosarcoma originally produced by inoculation of the DNA simian virus 40 (SV 40) in newborn hamsters, the viral genome persists in a noninfectious state in all or almost all of the tumor cells. The similarity between these findings and bacterial lysogeny led to further studies on the behavior of SV 40 viral genome in these tumor cells, with special reference to the possible presence of infectious nucleic acid in these cells and to various procedures for inducing an increased frequency of production of infectious virus. These new studies are briefly reported in the present communication.

Materials and Methods.—Same as in previous communication.[2] We recently found that an earlier passage level (49) of the BS-C-1 cells is ten times less sensitive in detecting SV 40 virus than the later passages we have been using, and that passage 49 cells failed to detect SV 40 virus when the tumor extract contained enough to produce a positive result in 11 of 20 passage 120, BS-C-1 tubes.

Results.—Receovery of infectious virus from later transplant passages of the tumors and role of age of tumor on detection of virus: As our work continued, we were surprised to find the greater regularity with which infectious SV 40 virus was being recovered from the tumors in the later transplant passages (Table 1). An analysis of the data (Table 2) showed that infectious virus was rarely recovered from large tumors excised less than 4 weeks after transplantation or after the first appearance

TABLE 1

FREQUENCY OF RECOVERY OF MINUTE AMOUNTS OF SV 40 VIRUS FROM CONSECUTIVE TRANSPLANT PASSAGES OF SV 40 HAMSTER TUMORS

Transplant passage no.	No. tumors tested	No. yielded virus	Result of test for virus
19	1	0	0/5*
21	1	1	10/10
22	1	1	5/10
23	1	1	5/9
24	1	1	3/30
25	3	0	0/30; 0/20; 0/20
26	2	0	0/30; 0/20
27	1	1	7/16
28	4	3	7/10; 4/10; 1/20; 0/20
29	3	1	19/20; 0/10; 0/10
30	4	2	19/20; 8/20; 0/20; 0/20
31	4	1	8/20; 0/20; 0/19; 0/10
33	2	2	20/20; 15/20
34	1	1	20/20
35	1	1	20/20
37	2 (pooled)	Pool	19/20

* Each tube of BS-C-1 Cercopithecus monkey cells received extract of 30 mg of grossly nonnecrotic tumor tissue. Numerator = no. of tubes showing SV 40 virus; denominator = no. of tubes inoculated. Each fraction represents results of tests on a single tumor.

of a palpable mass when small numbers of cells were used for transplantation. Thus, contrary to expectation, the older the tumor the more often it was found to contain virus; and the greater regularity of finding virus in the tumors of the later passages was accounted for by the fact that we were using tumors that had been growing for 40 days or longer. It should be pointed out here that all tests for infectious SV 40 virus are regularly monitored for chance contamination by including in each test large numbers of uninoculated BS-C-1 tubes, which have their weekly changes of medium in the same room, and in no instance has virus been detected in any of hundreds of such control tubes. Moreover, the positive results are readily reproduced in repeat tests.

TABLE 2

Interval-days	No. tumors tested	No. yielded virus
16–27	8	1 (12.5%)
28–77	23	16 (69.6%)

TABLE 3

No. of cells used for production of tumor	No. of tumors tested	Per cent yielded virus
10^6–10^7	31	55
10^1	24	58
$10^{0.3}$	13	77

Our previous conclusion that all or almost all of the tumor cells carry the SV 40 viral genome, despite the minute amounts of virus that are recovered from tumor extracts representing many millions of cells, was based on the fact that tumors derived from at least 2 cells yielded infectious virus with even greater regularity than the tumors that were derived from the transplantation of a million or more cells (Table 3). Actually the higher frequency of virus isolation from tumors representing the progeny of 2 cells is also accounted for by the fact that they were allowed to grow longer before being tested for virus.

Source of minute amounts of virus found in tumor extracts: The centrifuged, frozen, and thawed extracts of many tumors yielded virus, while many millions of trypsinized, frozen, and thawed cells derived from an aliquot of the same tumor mince usually yielded none (Table 4). The possibility that virus released from an occasional tumor cell may then multiply in the normal connective tissues or blood

TABLE 4

DIFFICULTY OF RECOVERING SV 40 VIRUS FROM LARGE NUMBERS OF FROZEN AND THAWED TRYPSINIZED CELLS FROM VIRUS-YIELDING TUMORS

	Results of Test for Virus in			
	15% Centrifuged Extract		Trypsinized Cells (frozen and thawed 3 ×)	
Tumor no.	No. of cells × 10^6 represented in extract added to each tube	No. of tubes showing virus	No. of cells × 10^6 added to each tube	No. of tubes showing virus
1	2.6	19/20	1.0	1/10
			0.1	0/10
2	2.0	20/20	1.0	0/10
			0.1	1/10
3	1.4	9/16	1.0	0/18
4	0.97	27/29	2.7	0/10
5	0.7	20/20	1.0	0/10
			0.1	0/10
6	0.18	20/20	0.34	2/10
7	0.16	20/20	0.26	0/10
8	0.14	30/30	0.24	0/20
9	0.13	27/30	0.25	1/20
10	0.9	1/20	1.1	0/10
11	0.51	0/20	1.0	0/9
12	0.46	0/19	1.1	0/10

vessels of the tumor is not supported by our finding that there is no multiplication of infectious virus even in newborn hamsters after inoculation of very large doses of SV 40 virus which, after many months, leads to tumor development. It is also noteworthy, in this respect, that no neutralizing antibody was found by the very sensitive plaque reduction test in the sera of any of 11 hamsters with virus-yielding transplantable tumors. It would appear therefore that the minute amounts of virus found in the tumor extracts, which maximally is only $10^{3.3}$ TCD_{50} per gm, and usually much less, represent an accumulation of a very small number of infectious doses spontaneously released by only an occasional cell out of many millions bearing the SV 40 viral genome. For comparison it should be noted that tissue cultures of the sensitive Cercopithecus BS-C-1 cells produce at least 100 infectious doses of virus per single cell.

Absence of infectious nucleic acid in virus-yielding tumors: One of the important questions is whether the SV 40 viral genome in the tumor cells exists in the form of infectious nucleic acid which, except in a rare tumor cell, cannot be converted into complete infectious virus. Nucleic acid was extracted by the cold phenol method from 3 gm of two tumors and the extract from a total of 1.5 gm (or about 1.7×10^8 tumor cells) was immediately tested on appropriately treated BS-C-1 cells in 20 roller tubes and 10 plates. No infectious virus was detected by this procedure, while simultaneous tests on the usual 15 per cent centrifuged extract from an aliquot of the same tumors yielded 2,000 TCD_{50} of infectious virus per gm in the roller tube test and 746 plaque-forming units per gm in the cells under agar. Gerber and Kirschstein[3] have also reported their inability to recover infectious nucleic acid from SV 40 virus-induced hamster ependymomas.

Induction of Increased Frequency of Production of Infectious Virus by Various Procedures.—Induction by propagation of tumor cells in vitro: We have previously reported[2] that in primary cultures large numbers of tumor cells either failed to release virus or did so in single bursts of minute amounts on rare occasions, while prolonged propagation and serial passages induced the release of somewhat larger amounts of virus and with greater frequency. Our earlier tests were done with

stationary cultures. Subsequent studies indicated that, while the tumor cells grew more profusely and were in better condition in stationary than in rolled cultures, there was a much greater frequency of production of small amounts of virus in primary cultures that were rolled (see data in Table 5).

TABLE 5

INFLUENCE OF ROLLING CULTURES OF SV 40 TUMOR CELLS ON SPONTANEOUS APPEARANCE OF INFECTIOUS VIRUS

No. of BS-C-1 tubes showing virus out of 5 inoculated with fluid removed from SV 40 tumor cultures on indicated day

Days after planting	Experiment 1					Experiment 2					
	Stationary (Bottles)†	Rolled (Tubes)‡				Stationary (Tubes)			Rolled (Tubes)		
		A	B	C	D	A	B	C	A	B	C
2	0	0	0	0	0	0	0	0	0	0	0
4	0	5	0	0	2	0	0	0	0	0	0
6	0	0	0	0	2	0	0	0	0	0	0
8	0	0	0	2	1	0	0	0	0	0	0
10	0	2	0	0	5	0	0	0	0	0	0
12	4	3	1	2	2	0	0	0	1	1	0
14	0	4	3	0	0	0;0*	0;0*	0;0*	0;0*	0;0*	3;0*
16	0	0	2	0	0						
18	0	5	0	0	5						
20	0;0*	4;3*	4;0*	5;4*	3;5*						

* Results of test on cells frozen and thawed 3 ×.

† Each of 2 bottles received 5 × 10^6 trypsinized tumor cells in 10 ml of medium which was changed every 2 days, pooled, and frozen for subsequent virus tests.

‡ Each of a group (designated A, B, etc.) of 5 tubes received 1 × 10^6 ml of the same lot of trypsinized tumor cells in 2 ml of medium. The tubes were left stationary for 2 days to allow attachment of the cells to the glass and were then transferred to a roller drum. The medium from each group of 5 tubes, completely changed every 2 days, was pooled and frozen for subsequent virus tests.

Induction by association of viable tumor cells with sensitive indicator (BS-C-1) cells in tissue culture: Gerber and Kirschstein,[3] working with an SV 40 virus-induced, hamster ependymoma in which they were unable to demonstrate infectious virus by tests on tumor extracts, frozen and thawed trypsinized cells, or in fluids from tumor cells in culture, made the important observation that minute amounts of infectious virus were regularly demonstrable when viable tumor cells (3,000–30,000 or more) were added to primary cultures of Cercopithecus kidney cells. Recently, Gerber[4] reported that minute amounts of virus were also recovered when the viable tumor cells were left in contact with primary rabbit kidney cells, which are not infected by intact SV 40 virus but may be infected by the DNA from the virus. Since our line of SV 40 tumor cells occasionally yielded minute amounts of infectious virus during growth on glass, we decided to study the phenomenon of "induction by association with sensitive cells" under conditions that were not conducive to proliferation of the tumor cells, as well as under conditions that permitted proliferation (see Table 6). In a medium (199 + 2 per cent calf serum) suitable for the maintenance and slow growth of the susceptible, BS-C-1 indicator cells, the tumor cells failed to grow and failed to attach themselves to the BS-C-1 cells except at the edge of the cell sheet where the tumor cells settled on the glass, even when 10^6 cells were added per tube; and yet infectious virus appeared in tubes that received 10^5 tumor cells and on occasion also smaller numbers of cells. The experiment shown in Table 6 indicates that a tumor, which by our sensitive method of virus detection failed to yield infectious virus either in a centrifuged extract or in millions of trypsinized, frozen, and thawed cells, nevertheless yielded virus when $10^{4.4}$ viable, trypsinized cells were added to the sensitive BS-C-1 cells. With a medium that supports the growth of tumor cells on glass, we found attachment and

TABLE 6

INDUCTION OF INCREASED FREQUENCY OF DEVELOPMENT OF INFECTIOUS SV 40 VIRUS BY CONTACT OF HAMSTER TUMOR CELLS WITH SUSCEPTIBLE CERCOPITHECUS MONKEY CELLS IN TISSUE CULTURE

Experiment 1. Medium used in this experiment is adequate for maintenance and slow growth of the susceptible cells but not for maintenance or growth of the tumor cells.

Tumor material added to tubes of susceptible cells	No. of tumor cells/tube	No. of tubes showing virus
15% centrifuged extract of tumor tissue	...	0/19
Trypsinized cells—frozen and thawed 3 ×	10^6	0/10
" " —viable	10^5	5/5
	$10^{4.4}$	4/5
	$10^{4.1}$	0/5

Experiment 2. (1) Effect of medium (B) suitable for continued growth of tumors cells. (2) Effect of exposing tumor cells to damaging doses of X rays on frequency of induction.

Tumor material added to tubes of susceptible cells	Medium used	No. of tumor cells/tube	No. of tubes showing virus
15% centrifuged extract of tumor tissue	A*	...	42/42
Trypsinized cells—frozen and thawed 3 ×	"	10^6	1/28
		10^5	0/10
" " – viable	"	10^6	4/5
	"	10^5	5/5
	"	10^4	0/5
	B	10^6	5/5
	"	10^5	4/4
	"	10^4	4/5
" " X ray 400 r	A	10^5	1/5
	"	10^4	0/5
	B	10^5	4/4
	"	10^4	4/5
" " X ray 1,600 r	A	10^5	2/5
	"	10^4	0/5
	B	10^5	4/4
	"	10^4	3/5

* Medium A is inadequate for maintenance or growth of the tumor cells. In tubes containing only this medium without susceptible cells, 10^6 or 10^5 tumor cells degenerated in a few days without yielding any virus.

growth of the tumor cells on the glass at the periphery of the BS-C-1 cell sheet and in open spaces between the BS-C-1 cells, when 10^5 or larger numbers of tumor cells were added. However, even in such a medium, 10^4 tumor cells added to BS-C-1 cells yielded virus without any evidence of growth or persistence of the tumor cells. It is evident therefore that induction of production of infectious virus by association with sensitive cells does not depend on proliferation of the tumor cells. Moreover, destruction of the proliferative capacity of the tumor cells by exposure to a 1,600 r dose of X ray did not abolish the capacity of 10^4 cells to yield infectious virus (see Table 6). In this experiment, the same number of irradiated cells planted on glass in the good growth medium quickly degenerated and failed to yield virus. Since in all of these tests the late appearance of the virus effect in the BS-C-1 indicator cells suggested that at most only 1–10 infectious doses of virus appeared during the process of induction even when 10^5 or more tumor cells were added, we attempted to determine the number of infectious foci that would appear when 10^6 viable tumor cells were planted on complete sheets of BS-C-1 cells in plastic plates and, after a 2 hr period of contact, were covered by a suitable nutrient agar overlay. No infectious foci appeared in any of 10 plates, although a simultaneous test in BS-C-1 cells maintained in medium 199 + 2 per cent calf serum in roller tubes yielded virus even in the tubes to which only 10^4 tumor cells were added. The efficiency of the BS-C-1 cell sheets on the plates to detect SV 40 virus of tumor origin was indicated

by the fact that the 15 per cent centrifuged extract from the same tumor tested at the same time yielded 112 plaque-forming units per ml. It is evident from this experiment that mere contact of viable tumor cells with sensitive BS-C-1 cells is not enough to induce production of infectious virus. Since extracts of millions of SV 40 viral genome-bearing tumor cells have failed to yield infectious nucleic acid, there is no basis for assuming that induction, when it occurs, represents a transfer of infectious nucleic acid from a tumor cell to a sensitive indicator cell capable of synthesizing complete virus. At the moment, the possibility must also be considered that something may be transmitted from a sensitive cell to a tumor cell which then induces it to produce not much more than a single or, at most, a few infectious particles of virus. As a matter of fact, in one preliminary experiment we found that addition of concentrated, frozen, and thawed extracts of BS-C-1 sensitive cells to growing tumor cells yielded traces of infectious virus in each of 2 bottles, while none appeared in 4 control bottles. Further experiments are needed to confirm this observation and to elucidate the mechanism of the interesting phenomenon of induction by association with sensitive cells.

Induction by X ray: Since some, although not all, lysogenic bacteria can be induced to synthesize infectious virus by exposure to appropriate doses of X ray under suitable conditions, we studied the effect of X irradiation on production of infectious virus by our line of SV 40 tumor cells. We used a Westinghouse Quadrocondex X-ray unit, operated at 250 kvcp, 15 ma, 0.5 mm Cu + 1 mm Al added filter, 1.85 mm half-value layer. We are indebted to Dr. James G. Kereiakes for determining the X-ray dosage under the conditions of our tests. The actual exposure was at a rate of 115 r per min, while the actual absorbed dose was 1.45 higher or 167 rads per min based on the data of Morkovin and Feldman.[5] The cells were irradiated in 160 ml pyrex glass bottles, either in suspension or after growth on the glass. The fluid medium was removed immediately after irradiation and replaced by fresh medium. Preliminary tests in which tumor cells, either in suspension or after 2 days of growth on glass, were irradiated with 400 or 1,600 r under medium 199 + 2 per cent calf serum, yielded negative results, but the control cells exposed to this medium grew very poorly and fell off the glass. In the experiment shown in Table 7, Eagle's minimum essential medium (MEM) + 30 per cent calf serum was used. The results indicate both an increased frequency and increased quantity of virus production in the irradiated bottles, which was most marked in the cells exposed to 3,000 r. Although exposure to 727 r ultimately resulted in disappearance of the original growth, a small number of new clones of cells emerged. These new cells had a different growth pattern from the original tumor cells. When enough of the 727 r resistant cells had been grown to permit another experiment, it was found that most of them were resistant to 727 r but were destroyed by exposure to 1,500 r or larger doses. However, as the data in Table 8 show, exposure to 364 r now failed to increase virus production, while a dose of 727 r appeared to be effective. It should be noted that the amount of virus produced was in all instances small, suggesting that only a small proportion of the cells were affected. It is possible that only a small proportion of tumor cells are inducible by X ray, and that this proportion may vary in different tumors. In an experiment with another tumor, in which the unirradiated cells yielded no virus in any of 4 bottles, irregular results were obtained; isolated bursts of virus release occurred only in bottles exposed to 364 r, 3,000 r, and

TABLE 7

INCREASED PRODUCTION OF INFECTIOUS SV 40 VIRUS AFTER IRRADIATION OF SV 40 TUMOR CELLS IN TISSUE CULTURE*

Days after X ray	No. TCD$_{50}$ of virus per bottle just before (i.e., 2 days after planting) and at indicated times after exposure to indicated dose of X ray						
	No. X ray controls	80 r 116 rad	159 r 231 rad	364 r 528 rad	727 r 1,054 rad	1500 r 2,175 rad	3000 r 4,350 rad
Just before	0	20	0	20	0	0	0
2	0	25	25	32	140	0	32
4	0	50	32	160	0	32	100
6	0	0	0	32	32	32	5,000
8	0	20	50	50	320	170	70
10	63	0	20	140	200	70	25
12	0	0	160	120	500	70	160
14	0	0	0	70	70	32	0
16	0	20	0	70	125	0	0
18	0	0	0	32	320	0	0
18 (Cells frozen and thawed 3 ×)	0	0	10	0	16	0	0
Total TCD$_{50}$ virus recovered during 8 days after X ray	0	95	107	274	492	234	5,202
Total TCD$_{50}$ virus recovered during 20 days after planting	63	135	297	726	1,743	406	5,387

* 5×10^6 tumor cells planted in Pyrex glass bottles with growth area of 37 cm^2. Two days later groups of 2 bottles received indicated doses of X ray, and 2 bottles remained unirradiated. The fluid growth medium (10 ml per bottle) was decanted every 2 days and replaced with fresh medium.

Note on growth of tumor cells. Two days after planting, just before irradiation, there was a profuse growth of flattened spindle-shaped cells in all bottles. *2 days after irradiation:* good, interlacing growth of spindle-shaped cells, seemingly somewhat more extensive in all the irradiated bottles. *4 days:* metabolism (judged by pH of fluid medium) was equally good in all, and the growth appeared equally good in the controls and those exposed to 80–727 r, while in the 1,500 and 3,000 r bottles a portion of the cells appeared degenerated and some had come off the glass. *6 days:* metabolism still equally good in all bottles; in the control, 80, and 159 r bottles the growth was heavy with many grossly visible tumors; in the 364 r bottles growth was extensive with very few tumors, and in the 727 r bottles growth was sparse with only a rare tumor; in the 1,500 and 3,000 r bottles the number of cells was less than in the 727 r bottles, and there were no tumors. *8 days:* metabolism still similar in all, but in the 1,500 and 3,000 r there were few cells left on glass and most of these appeared degenerated. *10 days:* metabolism similar in control, 80, and 159 r bottles with large numbers of small tumors (about 0.5 mm) and about 70% of the cells in between the tumors now round instead of spindle-shaped; 364 r—metabolism similar to controls, but growth less and few tumors; 727 r—metabolism less, and mixture of good sparse growth and many distorted, abnormal cells; 1,500 and 3,000 r—metabolism less than in others and only a few round cells, giant cells, and otherwise distorted cells left on glass. *12 days:* controls and 80–364 r bottles similar in metabolism, and progressive increase in number of tumors and round cells, except that number is less in 364 r bottles; 727 r—degenerated cells and giant cells, and in their midst clones of newly multiplying, good-looking fibrobasts; 1,500 and 3,000 r—little if any metabolism, very few cells on glass, less in 3,000 than in 1,500 r. *18 days after irradiation:* controls, 80 to 364 r, good metabolism and extraordinarily heavy growth, full of little tumors; 727 r—many new colonies of good-looking spindle-shaped cells and no tumors; 1,500 and 3,000 r—no evidence of metabolism and only rare round cell left on glass. After removal of the medium, 5 ml of fresh medium was added to all bottles, and the cells were frozen and thawed 3× to test for presence of intracellular infectious virus.

12,000 r but not in those exposed to 727, 1,500, or 6,000 r. Further experiments are needed to establish the effectiveness of X irradiation in increasing the frequency of production of infectious virus by the SV 40 tumor cells.

Specific Complement-Fixing Antigen in SV 40 Tumors.—Huebner *et al.*[6] recently reported that hamsters bearing "virus-free" transplantable tumors originally produced by adenovirus type 12, develop after a period of 4 weeks or more complement-fixing (CF) antibodies for adenovirus type 12 but not for the antigen common to all types of adenovirus, for which antibodies are found in human beings and animals infected with any type of adenovirus. At the symposium on virus-cancer relationships during the 1963 Annual Meeting of the National Academy, Huebner[7] reported the presence of specific antigens not only in adenovirus hamster tumors but also in SV 40 hamster tumors. The important issue here is whether the viral genome, which is transmitted in the tumor cells without the production of infectious

TABLE 8

EFFECT OF IRRADIATION ON APPEARANCE OF INFECTIOUS SV 40 VIRUS IN PROGENY OF 727 R RESISTANT TUMOR CELLS

X ray dose	Bottle no.	No. of BS-C-1 tubes showing virus out of 5 inoculated with fluid removed from tumor cell cultures on indicated day after X ray: Just before X ray	2	4	6	8	10	12
None	1	0	*1*	0	0	0	0	0
	2	0	0	0	0	0	0	*2*; 0*
Controls	3	0	*1*	0	0	*1*	0	0
	4	0	0	0	0	0	0	0; 0*
159 r	5	0	0	*3*	0	*1*	0	not tested
231 rad	6	0	0	*1*	0	*1*	0	0
364 r	7	0	0	0	0	0	0	*1*; 0*
528 rad	8	0	0	0	0	0	0	0; *1**
727 r	9	0	0	0	*3*	0	0	*4*; 0
1,054 rad	10	0	0	0	*1*	*1*	*1*	*3*; *4*
1,500 r	11	0	0	*1*	*5*	*5*	0	0; 0
2,175 rad	12	0	0	0	*1*	*1*	0	0; 0
3,000 r	13	*3*	0	*1*	0	0	*2*	0; 0*
4,350 rad	14	0	0	0	0	2	0	0; *1**
6,000 r	15	0	0	*2*	*5*	*3*	0	0; 0*
8,710 rad	16	0	0	*4*	*4*	*5*	*3*	0

* Refers to test on cells frozen and thawed 3×.

Note on growth of cells: Progressively heavier fibroblastic growth until termination of the experiment in control bottles and those exposed to 159 and 364 r. Bottles exposed to 727 r also showed progressive growth but less than others. Bottles exposed to 1,500–6,000 r showed no progressive growth] beginning with 2 days after X ray; at *4 days after X ray* many of the cells had fallen off the glass in bottles 11 to 16; at *6 days* there was no evidence of significant metabolism in bottles 11 to 16, and in bottle 15 there were only a small number of degenerated cells left on the glass and a somewhat larger number in bottle 16. At *8 days* after X ray, the growth was off the glass in bottles 11 to 16, except for isolated degenerated cells which were still adherent.

virus, is capable of ordering the synthesis of other antigens that are present in the virus particle or whether the specific antigen is not actually a component of the virus particle.

Our own preliminary tests on transplanted SV 40 tumors in adult hamsters yielded extracts which were either too anticomplementary for use even after centrifugation at 10,000 rpm for 1 hr, or failed to react with sera from tumor-bearing hamsters. However, an SV 40 tumor antigen obtained from Dr. Huebner was not significantly anticomplementary even after only light centrifugation and fixed complement with our hamster sera. When we followed Huebner's procedure of using only tumors transplanted in newborn hamsters and excised 7–10 days later, we were also able to obtain satisfactory potent antigens which reacted well with the sera from all the hamsters bearing transplanted SV 40 tumors, but not with sera from hamsters bearing polyoma or the Fortner fibrosarcoma no. 3 (F. Sa. no. 3) tumors. Sera from hamsters that had been inoculated as adults with a single large dose of SV 40 virus, which gives rise to the specific transplantation resistance phenomenon, also failed to react (see Table 9). Some of the sera from the SV 40 tumor-bearing hamsters failed to react with similarly prepared polyoma tumor and F. Sa. no. 3 tumor antigens, while others reacted with these antigens in lower titer. It is noteworthy, however, that neither the polyoma tumor nor the F. Sa. no. 3 tumor-bearing hamsters reacted with antigens prepared from their own tumors.

The data in Table 9 also show that sera from SV 40 tumor-bearing hamsters obtained 4 weeks or longer after transplantation or after first appearance of the tumor also react specifically with an antigen present in susceptible, BS-C-1 Cercopithecus cells infected with SV 40 virus. Hamsters bled earlier (see hamster Nos. P30-3 and P31-2 in Table 9) were found to react with the antigen contained in

TABLE 9

COMPLEMENT-FIXING ANTIBODIES IN SERA OF SV 40 TUMOR-BEARING HAMSTERS FOR ANTIGENS IN SV 40 VIRUS-INFECTED CERCOPITHECUS (BS-C-1) CELL CULTURES AND IN SV 40 HAMSTER TUMORS

	Hamster		C-F Titer with Indicated Antigen				
Sera	No.	Time bled (days)	BS-C-1 SV_{40} virus	BS-C-1 control	SV_{40} tumor	Polyoma tumor	F. Sa. no. 3 tumor
SV 40 tumor-bearing hamsters	P30-3	23[a]	<8	<8	64	<8	<8
	P31-2	28	<8	<8	16	<8	8
	P31-4	28	32	<8	64	16	32
	P30-5	37	8	<8	32	<8	<8
	1918	40	8+[b]	<8	256+?	<8	<8
	P34-2	61	32	<8	128	64	32
	1920	76	8+[b]	<8	256+?	8+[b]	not tested
	P25-4	77	64	<8	128	8	16
	Pool 1[c]	30+	160	<8	320+?	<20	not tested
Adult hamsters inoculated with $10^{7.7}$–10^{8} TCD_{50} SV 40 virus	1123[d]	62[f]	<8	<8	<8	<8	<8
	1218[e]	106	<8	<8	<8	<8	<8
	1240	144	<8	<8	<8	<8	<8
	1246	148	<8	<8	<8	<8	<8
	1217	148	<8	<8	<8	<8	<8
Polyoma tumor-bearing hamsters	P76-3	26[a]	<8	<8	<8	<8	16
	P75-5	31	<8	<8	<8	<8	<8
F. Sa. no. 3 tumor-bearing hamsters	P142-4	29[a]	<8	<8	<8	<8	<8
	" -5	29	<8	<8	<8	<8	<8
Cercopithecus monkey post-SV 40 virus infection[g]			128	<8	32	8	16
Normal Cynomolgus	1		<8	<8	32	32	32
	2		<8	<8	64	64	64
	3		<8	<8	32	32	32

[a] This refers to number of days after transplantation or after first observable tumor growth.
[b] Insufficient serum for quantitative tests.
[c] This serum pool, kindly provided by Dr. Robert J. Huebner, was derived from hamsters bearing transplantable SV 40 tumors derived from another source than our own.
[d] Hamster 1123 also had a fibrosarcoma no. 3 tumor (F. Sa. no. 3—Fortner) which followed inoculation of 10 tumor cells 43 days before bleeding; the tumor appeared about 3 weeks before bleeding, and measured 3.5 × 3.2 × 2 cm at the time of bleeding.
[e] Hamster 1218 also had a polyoma tumor which followed inoculation of 10 tumor cells 64 days before bleeding; the tumor appeared 43 days before bleeding, and measured 8.5 × 6 × 5 cm at the time of bleeding.
[f] This refers to number of days after injection of virus.
[g] This serum was kindly supplied by Dr. J. L. Melnick.
Note: The sera were heated at 60°C for 20 min. At least 1.5, but somewhat less than 2, exact units of complement, determined by titration in presence of antigen, were employed in these tests. The mixtures consisting of 0.2 ml serum, 0.2 ml antigen, and 0.2 ml complement were incubated for about 18 hr at 4°C.

the SV 40 tumor but not with the antigen in the SV 40 virus-infected BS-C-1 cells. The possibility that the later development of antibody to the antigen in the virus-infected BS-C-1 cells may represent a response to the continuous slow release of minute amounts of infectious virus by the SV 40 tumors is not supported by the fact that we failed to find neutralizing antibodies in the sera of 11 hamsters with virus-yielding tumors at the time they were bled after 40–76 days of tumor growth. Such hamster sera, as well as others without neutralizing antibodies from hamsters whose tumors did not yield virus, had antibodies for both the SV 40 tumor antigen and the SV 40, BS-C-1 culture antigen.

Our present data do not permit any decision whether (a) animals in which SV 40 virus multiplies without producing tumors also develop CF antibody for the tumor antigen—because the single available Cercopithecus monkey postinfection serum reacted nonspecifically with all the tumor antigens as did the 3 normal Cynomolgus monkey sera which had no antibody for the SV 40 virus, BS-C-1 antigen, and (b) the CF antibody in the SV 40 tumor-bearing hamster sera which reacts with the antigen in SV 40 virus-infected BS-C-1 cells is directed against an antigenic compo-

nent of the virus particle or a specific by-product of the infected cell. One point seems clear, however, viz., that the specific resistance to transplantation of SV 40 tumor cells induced by inoculation of adult hamsters with large doses of SV 40 virus[8-10] is not associated with either of these CF antibodies. Further work with purified viral antigens will have to be done to establish whether the SV 40 tumor cells carry genetic information for the synthesis of at least a portion of the viral antigenic material.

Summary.—In transplanted SV 40 hamster tumors spontaneous development of infectious virus occurs only in an occasional cell out of many millions that carry the viral genome in a noninfectious state, more frequently in older tumors than in those that have grown for less than 4 weeks. No infectious nucleic acid capable of producing complete SV 40 virus in susceptible Cercopithecus cells was found in the tumor cells. Induction of a greater frequency of synthesis of infectious virus in tumor cells has now been demonstrated by three procedures: (1) propagation of the tumor cells *in vitro*—more frequently in rolled than in stationary cultures, although the latter are more suitable for optimum growth of the tumor cells; (2) by association of viable tumor cells with susceptible cells under certain conditions of contact which do not require either proliferation or persistence of the tumor cells; (3) by an X-ray effect on a very small proportion of the tumor cells.

SV 40 tumors transplanted in newborn hamsters yield a satisfactory complement-fixing antigen which reacts specifically only with the sera of hamsters bearing SV 40 tumors but not with sera of hamsters bearing either polyoma tumors or Fortner fibrosarcoma no. 3 tumors. Hamsters bearing transplanted SV 40 tumors for 4 weeks or longer also develop complement-fixing antibody for an antigen present in BS-C-1 Cercopithecus cells infected with SV 40 virus, although these sera contain no neutralizing antibodies. Adult hamsters inoculated with large doses of SV 40 virus, which induces a specific resistance to transplantation of SV 40 tumor cells, possess no antibody for the antigen in the tumor or the antigen in the SV 40 virus-infected Cercopithecus cells. The available data are not sufficient for a conclusion that the noninfectious viral genome in the SV 40 tumor cells carries information for the synthesis of at least a part of the viral antigen, although it gives rise to antigens that are specific for the SV 40 virus.

The data on induction of infectious virus in the SV 40 tumor cells provide a new approach to the search for etiologically significant, dormant viruses in human cancers.

* The basic data in this communication were presented by Dr. Sabin at the Symposium on Virus-Cancer Relationships, *Chairman*, Wendell M. Stanley, during the 100th Annual Meeting of the National Academy of Sciences, under the title "Continuous Transmission of Noninfectious SV 40 Viral Genome in Hamster Cells." Supplementary information which was incomplete at the time of the Symposium presentation has been added in proof.

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*GENETIC TRANSFORMATION AMONG LIVING PNEUMOCOCCI IN THE MOUSE**

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Since the discovery of genetic transformation[1] and the identification of the pneumococcal transforming principle as deoxyribonucleate,[2] the occurrence and significance of transformations in nature have been of interest. Until recently, however, transformations were observed to occur only under artificial conditions. For transformations to take place in a living host, large numbers of heat-killed cells (10^9–10^{10})[1, 3, 4] or a large amount of partly purified DNA-protein had to be provided as DNA source.[5] For *in vitro* transformations, purified deoxyribonucleate was usually employed. In 1951 Hotchkiss reported that penicillin-lysed pneumococcal cells in relatively small numbers could be used as DNA donors in transformation experiments *in vitro*.[6] Recent experiments[7] have shown that growing unmodified pneumococcal cultures release genetically active DNA and that recombinants can be recovered from mixed, growing cultures in the test tube. This work lent support to the idea that genetic exchange via transformation may be a natural recombination mechanism. The present experiments were carried out to determine whether genetic exchange can take place between two genetically distinguishable populations of pneumococci growing together in the same living host, the mouse.

Materials and Methods.—Pneumococcal strains: R36NC Sm^r, a rough, unencapsulated strain derived originally from an encapsulated type II strain, D39S[2], and made resistant to a high level of streptomycin (1,000 γ/ml) by transformation. This strain is relatively avirulent for mice (Table 1). Strain IIIS-IR6 was obtained by transformation of a rough variant from a type I strain I/519/43/41[8] with DNA from a fully encapsulated type III strain, IIIS-A/66. Strain IIIS-IR6 synthesizes type III polysaccharide, but in small amount. Colonies on blood agar are smooth but are much smaller than naturally occurring, virulent type III. The mouse virulence of strain IIIS-IR6 is very low (Table 1).

TABLE 1

VIRULENCE OF STRAINS OF PNEUMOCOCCI FOR MICE

Culture	Original derivation	Colony type	Capsular type	Streptomycin resistance	Intraperitoneal virulence in mice*
DNA donor (IIIS-IR6)	Type I	Small, smooth	III	−	2×10^7
Recipient (R36NC Sm^r)	Type II	Rough	R	+	2×10^8
Transformant (IIIS-R36NC Sm^r)		Large, smooth	III	+	2×10^2

* Number of cells required to kill mice following intraperitoneal injection.

Transformation system: When R36NC Sm^r is transformed by DNA from IIIS-IR6 *in vitro*,[3] the transformants (IIIS-R36NC Sm^r) are fully encapsulated type III cells and form large, mucoid

colonies typical of naturally occurring, virulent type III strains. This transformed strain is far more pathogenic for mice than either parental strain (Table 1) and in addition is streptomycin resistant. The transformant (IIIS-R36NC Sm^r) can be distinguished from IIIS-IR6 by its virulence, its ability to synthesize large amounts of type III polysaccharide, and by its resistance to streptomycin. If strain IIIS-IR6 were to mutate to production of large amounts of capsule, the chances of a simultaneous mutation to streptomycin resistance would be extremely small. If R36NC Sm^r, originally derived from type II, were to revert to capsule synthesis (an event never observed during 25 years of its use in the laboratory), the type of capsule synthesized would be type II, whereas the transformant produces type III capsule. The system used provides, therefore, (1) reliability in identifying the transformed type; (2) a selective advantage (higher virulence) of the transformed type over both parental types *in vivo*.

Transformation method: Mice of strain CFW, weighing 18–20 gm, were injected intraperitoneally. The cultures to be used in transformation experiments were incubated overnight at 37°C in 1% neopeptone-meat infusion broth containing 2% fresh defibrinated rabbit blood.[2] Each culture was then diluted 1:1000 in modified Adams and Roe medium[7] and incubated 3 hr at 37°C, then cooled to 25°C for 15 min to increase transformability.[9] At the end of this time, the donor culture IIIS-IR6 had reached a population density of about 4×10^8 colony-forming units per ml. It was placed in a 0°C bath until injected into mice. At the same time, the recipient culture, R36NC Sm^r, had reached a population density of about 2×10^7 colony-forming units per ml. It was concentrated tenfold by filtration through a Millipore filter, porosity $0.45 \pm 0.02\ \mu$ (Millipore Filter Corporation), and resuspended in fresh, modified Adams and Roe medium of one tenth the original volume to reach about 2×10^8 colony-forming units per ml. Each mouse was injected intraperitoneally with 10^8 R36NC Sm^r cells (0.5 ml) and 2×10^6 IIIS-IR6 cells (0.5 ml) in sequence, using different syringes and needles for each culture.

The control series was treated in an identical manner except that pancreatic deoxyribonuclease (1 X recrystallized-Worthington) in final concentration of 10 γ/ml and $MgSO_4 \cdot 7H_2O$ (0.007%) were added to each culture prior to injection.

Detection of transformation: Under the above conditions about three fourths of the injected mice died within 24–48 hr. A loop of heart blood from each mouse was streaked on BBL-Brain-Heart infusion agar containing 5% horse blood and 500 γ/ml streptomycin. In order to detect small numbers of transformants, a loop of heart blood was also inoculated into neopeptone blood broth containing 10 γ/ml deoxyribonuclease to prevent *in vitro* transformation from taking place. After overnight incubation at 37°C the blood broth cultures were streaked on horse-blood brain-heart infusion agar containing 500 γ/ml streptomycin sulfate (Eli Lilly and Co.). The capsular

TABLE 2

OCCURRENCE OF TRANSFORMATION IN MICE

Experiment	Cultures injected	Number of mice injected	Number of mice dead	Number of mice from which transformed type* was isolated
I	IIIS-IR6 + R36NC Sm^r	13	10	6†
II	IIIS-IR6 + R36NC Sm^r	10	8	6
	IIIS-IR6 + R36NC Sm^r + DNase	11	6	0
	IIIS-IR6 alone	11	0	0
	R36NC Sm^r alone	9	0	0
III	IIIS-IR6 + R36NC Sm^r	20	14	6
	IIIS-IR6 + R36NC Sm^r + DNase	21	18	0
IV	IIIS-IR6 + R36NC Sm^r	31	22	7
	IIIS-IR6 + R36NC Sm^r + DNase	32	26	0
V	IIIS-IR6 + R36NC Sm^r	9	5	1
	IIIS-IR6 + R36NC Sm^r + DNase	10	8	0
	Total (experimental)	83	59	26
	Total (DNase control)	74	58	0
	Total IIIS-IR6 alone	11	0	0
	Total R36NC Sm^r alone	9	0	0

* Transformed type = IIIS-R36NC Sm^r, large colony, mucoid type III, mouse virulent, streptomycin resistant.
† From the remainder of the dead mice, only the parental types were isolated. Though each parental type is not lethal by itself in the dose used, the mixture of the two has greater virulence than would be expected if each strain acted independently.

type of the smooth colonies was identified immunologically using the Quellung method and rabbit antisera.

Results and Discussion.—A total of five different experiments was performed. The results are presented in Table 2.

It is evident from Table 2 that, in 31% (26/83) of the mice which were injected with both donor and recipient cultures in the absence of exogenous DNase, at least one transformation event had taken place. It can also be concluded that genetic recombination was due to DNA-mediated transformation, since no recombination took place when DNase was added to the system.

These experiments demonstrate that DNA-mediated transformation can occur spontaneously in genetically mixed populations of pneumococci growing in a living host. A more detailed discussion of the significance of transformation as a natural method of recombination for some microbial species was presented previously.[7]

In addition to studies on pneumococcus and streptococcus, spontaneously occurring DNA-mediated transformation *in vitro* has since been observed in *B. subtilis*[10] and in *H. influenzae*.[11] It is probable therefore that genetic transformation may play a more widespread role in microbial recombinations in nature than has been supposed.

Summary.—DNA-mediated transformation occurs spontaneously among genetically-marked pneumococci growing together in a living host, the mouse.

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*STUDIES ON THE FORMATION OF TOBACCO MOSAIC VIRUS RIBONUCLEIC ACID, III. UTILIZATION OF RIBONUCLEOSIDES OF HOST RIBONUCLEIC ACID**

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The process by which TMV-RNA[1] replicates within the cell is not understood, either with regard to mechanism or site of formation. Even though its synthesis is not mediated by cell DNA,[2] it is still possible that the nucleus is the site of its

formation. Since the formation of considerable amounts of nucleic acid possessing the properties of TMV-RNA is not the normal function of the cell, the materials needed for its synthesis might not be present in adequate amounts in the host cell. Hence, the prerequisite for its formation is the availability of the adequate amounts of the building blocks, which could be provided either by *de novo* synthesis or by the degradation of the host RNA. The evidence in support of the latter was presented in the previous paper.[3] Following infection with TMV, microsomal RNA of the cytoplasmic fraction, which forms about 55–60 per cent of the total tobacco leaf RNA, is rapidly degraded and its degradation products are utilized in the synthesis of TMV-RNA. The evidence presented in this paper further demonstrates that the ribonucleoside moieties of the microsomal RNA are utilized in the synthesis of TMV-RNA.

Experimental.—TMV inoculum for infecting tobacco plants: The common strain of TMV used in this investigation was kindly supplied by Dr. F. O. Holmes of the Rockefeller Institute. TMV inoculum used for infecting the tobacco plants was prepared as previously described.[3]

Plants: Tobacco plants (*Nicotiana tabacum* var. Turkish) used in these studies were grown in a greenhouse. These were kindly provided by Dr. Armin Braun of the Rockefeller Institute.

Growth of infected tobacco plants in Hoagland's medium containing Pi^{32}: The tobacco plants, 4–5 wks old, were transferred from the greenhouse to a room in which the temperature was maintained at 26°. Plants of same size and general appearance were selected, and bottom leaves were removed and discarded. The remaining leaves were dusted with carborundum and were rubbed with TMV inoculum. The leaves were afterward rinsed with water. The plants were immediately removed from the pots, and their roots were washed with tap water. One plant was placed in each bottle containing 80 ml of Hoagland's nutrient solution to which was added Pi^{32} (amounts of Pi^{32} per ml of the nutrient solution are given in Tables 1 and 2). The phosphate content of the Hoag-

TABLE 1

SPECIFIC RADIOACTIVITIES OF MICROSOMAL RNA AND TMV-RNA

Nucleic acid	Counts/min/μg RNA	
	Expt. 1	Expt. 2
Microsomal RNA	194	162
TMV-RNA	784	740

Expt. 1, plants were grown in nutrient solution containing 0.6 μc Pi^{32} per ml; Expt. 2, plants were grown in nutrient solution containing 0.5 μc Pi^{32} per ml. Rest of the conditions are described in the text.

TABLE 2

SPECIFIC RADIOACTIVITIES* OF MONONUCLEOTIDES OBTAINED BY DIGESTION WITH ALKALI AND SNAKE VENOM PHOSPHODIESTERASE

Nucleotides	Experiment 1		Experiment 2	
	5′-	2′-(3′-)	5′-	2′-(3′-)
Cytidylic acid	224	237	218	235
Adenylic acid	243	223	232	231
Guanylic acid	235	224	215	216
Uridylic acid	238	218	221	232
Average	235	226	222	228

* Counts/min/mμmole nucleotide.
Expt. 1, plants were grown in nutrient solution containing 0.6 μc Pi^{32} per ml; Expt. 2, plants were grown in nutrient solution containing 0.5 μc Pi^{32}. Rest of the experimental conditions are described in the text.

land's nutrient solution was reduced to one tenth of that prescribed to ensure the maximum uptake of Pi^{32} by the plants. The nutrient solution was fortified by the weekly addition of 0.1 ml of 0.5% ferric tartrate to each bottle. The nutrient solution was gently aerated throughout the experimental period. The level of the solution in the bottles was maintained by the frequent addition of the nutrient solution containing Pi^{32}. The bottles were covered with black cloth to exclude light. The plants were illuminated 14 hr a day. After nine days, the leaves were har-

vested, and the P^{32}-TMV-RNA and P^{32}-microsomal RNA were isolated according to the procedures described below.

Determination of the radioactivities of microsomal RNA and TMV-RNA: Unless otherwise stated, all operations were conducted at 0–4°. Immediately after harvesting the radioactive tobacco leaves, their mid-ribs were removed, and the leaf blades were quickly cut into small pieces. Two gm portions were placed in a homogenizer tube of 15 ml capacity, fitted with a teflon pestle. The leaves were ground for 5 min with 5 ml of 0.5 *M* sucrose containing 0.01 *M* tris-HCl buffer at pH 8.0 and 0.002 *M* $MgCl_2$. The ground material was centrifuged for 20 min at 20,200 *g* in the refrigerated Servall centrifuge. The residue was washed twice with 3 ml of above grinding medium. The combined supernatants were centrifuged for 2 hr at 105,000 *g* in the No. 40 rotor of the Spinco Model L ultracentrifuge. After draining the supernatant thoroughly, the pellet was suspended in 1.5 ml of 0.1 *M* sodium phosphate buffer at pH 7.4 containing 4×10^{-3} *M* EDTA and centrifuged for 20 min at 12,100 *g* in the refrigerated Servall centrifuge. The residue was extracted once with 1.5 ml of the above phosphate buffer.

To the above combined extracts were added 3 ml of chloroform and 0.06 ml of octanol. The mixture was shaken at room temperature for 15 min and centrifuged in the clinical centrifuge for 10 min at top speed. The top aqueous layer, which was pale green and opalescent, containing TMV and microsomal RNA, was drawn out with a capillary pipette and centrifuged for two hr at 105,000 *g* in the No. 40 rotor of the Spinco Model L ultracentrifuge. The microsomal RNA, present in the supernatant, and the TMV, present in the pellet, were further purified as described below.

The ratio of light absorption at 280 mμ to 260 mμ of the supernatant was 0.48 in 0.1 *M* sodium phosphate buffer at pH 7.4. To this was added 0.3 ml of TMV antiserum (1 mg protein). After 15 min at 0°, the mixture was centrifuged for 15 min at 7,900 *g* in the refrigerated Servall centrifuge. There was a small amount of precipitate, which was noticed even with the extracts obtained from healthy tobacco leaves, at the bottom of the centrifuge tube. To the supernatant was added 0.66 ml of 40% TCA with mixing. After 15 min the mixture was centrifuged for 10 min at 7,900 *g* in the refrigerated Servall centrifuge. The precipitate was washed three times with 1 ml of cold 6% TCA and twice with absolute alcohol. The precipitate was finally washed once with ether at room temperature and air-dried.

The pellet containing TMV was extracted twice with 0.5 ml of 0.1 *M* sodium phosphate buffer at pH 7.4 containing 4×10^{-3} *M* EDTA. The supernatants, obtained by centrifugation for 15 min at 12,100 *g* in the refrigerated Servall centrifuge, were combined. To the combined extracts were added 0.1 ml of pancreatic RNAase (400 μg) and a drop of chloroform to prevent microbial growth. The mixture was incubated at room temperature for 24 hr. At the end of the incubation period, the tube was transferred to an ice bath and to this was added with mixing 0.6 ml of cold 40% TCA. After 15 min, the precipitate was recovered by centrifugation. It was washed and air-dried as described above.

The above air-dried precipitates containing microsomal RNA and TMV-RNA were dissolved in 0.3 ml of *N* NaOH, incubated at 30° for 24 hr, and neutralized with 2.0 *N* HCl. 0.4 ml aliquots of the neutralized hydrolysates were placed on Whatman No. 3 MM filter papers as narrow bands (10 cm long) and dried in a current of air at room temperature. The chromatograms were developed in the isopropanol-water-ammonia solvent system[4] for 24 hr, dried at room temperature, and the nucleotides were located under ultraviolet light. In this solvent system the alkali-hydrolyzed RNA gives two bands, one containing guanosine 2′-(3′-) phosphate and the other containing a mixture of 2′-(3′-) phosphates of adenosine, cytidine, and uridine. These two bands were eluted together by running distilled water through them for 4 hr. A blank filter paper of the same dimensions, which was cut out of Whatman No. 3 MM filter paper, subjected to the above chromatographic procedure, was likewise eluted. The eluates were made up to a suitable volume, and their absorptions were measured in the Beckman spectrophotometer at 260 mμ. A known amount of TMV-RNA, subjected to the above procedure, was used as a standard in calculating the RNA contents of the samples.

Aliquots of eluates containing the microsomal RNA and TMV-RNA nucleotides were dried on metal planchets and their radioactivities were measured.

Preparation of P^{32}-labeled TMV and TMV-RNA: Unless otherwise stated, all operations were carried out at 0–4°. Fifty gm of radioactive leaves were frozen immediately after harvesting.

The frozen material was placed in a large mortar and quickly powdered with a pestle. To the powdered material were added 10 gm of sand and 50 ml of 0.1 *M* sodium phosphate buffer at pH 7.4 containing 4×10^{-3} *M* EDTA. The mixture was ground for 5 min; to this were added 100 ml of the above buffer, and the grinding was continued for another 5 min. The ground material was centrifuged for 15 min at 17,300 *g* in the refrigerated Servall centrifuge. The residue was washed once with 50 ml of the above grinding buffer. The combined supernatants were centrifuged for 1 hr at 78,480 *g* in the No. 30 rotor of the Spinco Model L ultracentrifuge. The pellets were frozen for 30 min at −20° and then they were soaked overnight at 4° covered with phosphate buffer used for grinding. The virus was isolated from the combined extracts by four cycles of differential centrifugation; the low speed cycles consisted of a 15-min centrifugation in the refrigerated Servall at 17,300 *g*, and the high speed cycles consisted of a 1-hr centrifugation at 105,000 *g* in the No. 40 rotor of the Spinco Model L ultracentrifuge. The pellets were always taken up in the phosphate buffer used for grinding the leaves and they were allowed to go into solution gradually. The final preparation, which was colorless and opalescent, was dialyzed for 24 hr against two changes of distilled water (4 liters) with continuous stirring. The yield of the virus was about 120–140 mg.

TMV-RNA was prepared from the purified TMV according to the procedure previously described.[5]

Determination of specific radioactivities of mononucleotides obtained by hydrolysis of P^{32}-TMV-RNA with alkali: One mg of P^{32}-TMV-RNA was dissolved in 0.5 ml of *N* NaOH, incubated at 30° for 24 hr, and neutralized with 2 *N* HCl. The rest of the procedure involving chromatography of the neutralized hydrolysate in isopropanol-water-ammonia solvent system, elution of nucleotides from the chromatogram, was exactly the same as described above.

The eluate from the chromatogram containing all four nucleotides was taken to dryness over anhydrous $CaCl_2$ in a vacuum desiccator at room temperature, and the residue was taken up in 0.05 *M* ammonium formate buffer at pH 3.4. A sample of 0.05 ml was placed as a narrow band at a distance of 10 cm from one end of the strip (9 × 55 cm) of Whatman No. 3 MM filter paper, which was wetted with 0.05 *M* ammonium formate buffer at pH 3.4 and blotted to remove excess buffer. This was subjected to electrophoresis for 3 hr using 0.05 *M* ammonium formate buffer at pH 3.4 and a current of 10 ma. This procedure separates the mixture of nucleotides into four bands: cytidine 2′-(3′-) phosphate, adenosine 2′-(3′-) phosphate, guanosine 2′-(3′-) phosphate, and uridine 2′-(3′-) phosphate. The paper strip was dried at room temperature. The bands were located under ultraviolet light, cut out, and eluted with 0.01 *N* HCl for 18–20 hr at room temperature. The amount of nucleotide present in each eluate was determined spectrophotometrically. An aliquot of each eluate was dried on a metal planchet, and the radioactivity was measured.

Determination of specific radioactivities of mononucleotides obtained by hydrolysis of P^{32}-TMV-RNA with snake venom phosphodiesterase: The snake venom phosphodiesterase used in these studies and the conditions of hydrolysis were the same as those of Sugiyama and Fraenkel-Conrat.[6] 0.3 mg of P^{32}-TMV-RNA was dissolved in 0.06 ml of 0.1 *M* borate buffer at pH 8.6 containing 0.005 *M* $MgCl_2$. To this solution were added three 0.02 ml aliquots (0.3 unit/0.02 ml) of snake venom phosphodiesterase at 0, 2, and 4 hr. The hydrolysis was carried out for 6 hr at 37°.

0.06 ml of the hydrolysate was subjected to electrophoresis at pH 3.4 for 3 hr according to the procedure described above. Under these conditions four nucleoside 5′-phosphates present in the hydrolysate separate in the same order as the nucleotides in the alkali-hydrolysates. The nucleotides were eluted from the electrophoretic strip, and their amounts and radioactivities were determined as described above.

Determination of the radioactivities of the dialyzable fragments formed during the degradation of P^{32}-TMV-RNA with snake venom phosphodiesterase: A reaction mixture containing 1 mg of P^{32}-TMV-RNA in 0.5 ml of 0.05 *M* borate buffer at pH 8.6, containing 0.0025 *M* $MgCl_2$ and 0.7 unit (0.05 ml) of snake venom phosphodiesterase, was dialyzed at 35° against 3 ml of above buffer containing $MgCl_2$. At 1-hr intervals the dialysis bag was transferred to a tube containing a fresh amount of 3 ml of the above buffer. The hydrolysis was continued for 14 hr with gentle shaking. The absorbancies of dialyzable fragments were measured at 260 mμ in the Beckman spectrophotometer, and their radioactivities were measured by drying a known amount on metal planchets. The material present in the dialysis bag at the end of the reaction period was made up to 3 ml

with the above borate buffer, and its absorbancy at 260 mμ and its radioactivity were determined.

Reagents: Pancreatic RNAase was purchased from the Worthington Biochemical Corp., Freehold, New Jersey, and sea sand from Merck and Co., Inc., Rahway, New Jersey. Pi^{32} was purchased from the Oak Ridge National Laboratory. Snake venom phosphodiesterase was kindly supplied by Dr. M. Laskowski, Sr., Marquette University Medical School, Milwaukee. TMV antiserum was kindly supplied by Dr. A. Siegel of the University of Arizona.

Results.—Specific radioactivities of microsomal RNA and TMV-RNA: The results presented in Table 1 show that the incorporation of P^{32} into microsomal RNA and TMV-RNA was significantly different. The small amount of radioactivity present in the microsomal RNA might represent a new synthesis of microsomal RNA or some species of RNA (template?) occurring associated with the microsomes. Experiments are in progress to elucidate this point. The amount of isotope incorporated into TMV-RNA was greater than that associated with microsomal RNA and this is not surprising because of its extensive synthesis in the cell following infection.

In the previous paper[3] evidence for the degradation of microsomal RNA following infection with TMV and utilization of its degradation products in the synthesis of TMV-RNA, was presented. If the phosphate moiety of TMV-RNA is derived entirely from the microsomal RNA, its specific radioactivity would have been the same as that of microsomal RNA. Since this is not the case, two possibilities concerning the source of its phosphorus can be suggested: one, the phosphate moiety of TMV-RNA is not derived from the microsomal RNA; and two, the phosphate moiety of TMV-RNA is partly derived from the microsomal RNA and partly from other sources. The latter possibility would give rise to nonuniformity in the distribution of P^{32} label in TMV-RNA. The evidence obtained from three different experiments (see below) suggests the remarkable uniformity in the labeling of the entire TMV-RNA molecule.

Uniformity in the labeling of P^{32}-TMV-RNA: The results presented in Table 2 show that the radioactivities of nucleoside 2′-(3′-) phosphates, obtained by hydrolysis of TMV-RNA with alkali, are similar. Since the synthesis of polyribonucleotides is accomplished by the polymerization of ribonucleoside 5′-phosphate moieties of ribonucleoside 5′-triphosphates, the phosphates appearing as the 2′-and 3′-nucleotides, obtained by alkali hydrolysis, could bear no metabolic relation with its associated base. For this reason, P^{32}-TMV-RNA was hydrolyzed with snake venom phosphodiesterase to obtain ribonucleoside 5′-phosphates, and the results presented in Table 2 show that the radioactivities of four nucleotides obtained in this manner are also similar. These results are at variance with those reported by Staehelin,[7] who observed heterogeneity in the distribution of radioactivity in the ribonucleoside 5′-phosphates. Further evidence in support of the uniformity in labeling of the entire TMV-RNA molecule is presented in Figure 1.

Since snake venom phosphodiesterase hydrolyzes nucleic acid stepwise[8] from the 3′-hydroxyl end of the chain,[9] it was thought that the degradation of P^{32}-TMV-RNA with this enzyme might give further information concerning the distribution of P^{32} label. The absorbancies and the radioactivities of the dialyzable fractions obtained as a result of digestion of P^{32}-TMV-RNA with snake venom phosphodiesterase are given in Figure 1. The absorbancy curve is almost superimposed on the radioactivity curve, suggesting the remarkable uniformity in the labeling

throughout the molecule. In 14 hr about 90 per cent of the RNA was rendered dialyzable. The specific radioactivity of the material that remained in the dialysis bag was the same as that of the dialyzable fractions, obtained throughout the experimental period.

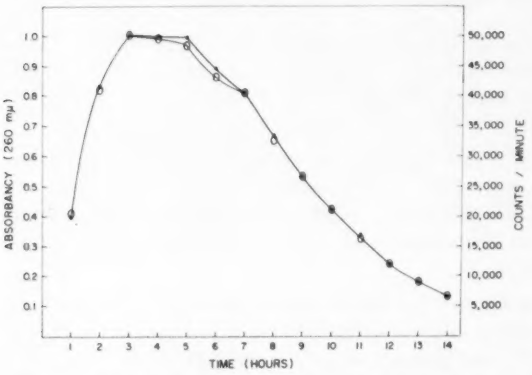

FIG. 1.—Distribution of radioactivity in P^{32} -TMV-RNA. O——O, absorbancy of the dialyzable fractions at 260 mμ; ●——●, radioactivity of the dialyzable fractions, counts/min. Experimental conditions are described in the text.

Discussion.—As shown in this paper, the radioactivity of TMV-RNA is more than four times that associated with the microsomal RNA, and it is uniformly distributed throughout the entire molecule. Hence, the phosphate moiety of TMV-RNA is not contributed by the microsomal RNA. The evidence for the degradation of microsomal RNA following infection with TMV and the utilization of its degradation products in the synthesis of TMV-RNA was presented in the previous paper;[3] the uridine moiety of the microsomal RNA was indeed utilized in the synthesis of TMV-RNA. On the basis of the results presented in this paper and the previous paper,[3] it can be concluded that only the ribonucleoside moieties of microsomal RNA are utilized in the synthesis of TMV-RNA.

The degradation of microsomal RNA might be effected by the combined actions of ribonuclease and phosphatase present in the tobacco leaf. In addition to the RNAase,[10–12] the properties of which were described previously, we have evidence for the occurrence of another nuclease in tobacco leaves.[13] The partial purification and properties of phosphatase present in tobacco leaves were previously described.[14] By the action of these three enzymes, microsomal RNA could be degraded to ribonucleosides, which after phosphorylation to nucleoside 5′-triphosphates could serve as substrates for the polymerizing enzyme involved in the synthesis of TMV-RNA. The mode of conversion of ribonucleosides to nucleoside 5′-triphosphates is being studied, and the results will be published elsewhere.

Summary.—The radioactivities of TMV-RNA and microsomal RNA, isolated from infected tobacco leaves grown in the presence of P^{32}, were determined. The specific radioactivity of TMV-RNA is more than four times that associated with the microsomal RNA. The specific radioactivities of four mononucleotides, obtained by the digestion of P^{32}-TMV-RNA with alkali and snake venom phosphodiesterase, are similar. Furthermore, the distribution of radioactive label in the entire molecule of TMV-RNA is remarkably uniform. Hence, the phosphate moiety of TMV-RNA is not derived from the microsomal RNA. On the basis of the results presented in this paper and the previous publication of this series, it is concluded that only the ribonucleoside moieties of microsomal RNA are utilized in the synthesis of TMV-RNA.

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[1] Abbreviations: TMV, tobacco mosaic virus; TMV-RNA, tobacco mosaic virus ribonucleic acid; DNA, deoxyribonucleic acid; EDTA, ethylenediaminetetraacetate; TCA, trichloroacetic acid; RNAase, ribonuclease.

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*THE EFFECT OF ACRIFLAVINE ON PHOTOREVERSAL OF LETHAL AND MUTAGENIC DAMAGE PRODUCED IN BACTERIA BY ULTRAVIOLET LIGHT**

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Mutations to prototrophy, induced in auxotrophic bacteria by far ultraviolet light (UV), may be eliminated by subsequent exposure to near UV or visible light (photoreversal),[1] or, in the dark, by temporary suppression of postirradiation protein synthesis ("dark repair").[2] It has been proposed[3] that photoreversal and "dark repair" of these mutations involve alternative mechanisms for the enzymatic repair of the same premutational damage produced by UV in the bacterial deoxyribonucleic acid (DNA). Since killing by UV is photoreversible, but not subject to "dark repair" (in *Escherichia coli* B/r), and since the same is true of certain UV-induced mutations other than those to prototrophy,[4] it seems certain that UV produces at least two kinds of photoreversible lesions in bacteria, only one of which is also subject to "dark repair."

Acriflavine has been shown to interfere with "dark repair" of UV-induced prototrophy, and it has been suggested that it does so by combining with and modifying the irradiated bacterial DNA in a way that reduces the accessibility of the premutational lesions to the postulated "dark repair" enzyme.[5] If the lesions leading to prototrophy are indeed different from those leading to death, acriflavine might be expected to interfere also with photoreversal of these mutations, but not necessarily with photoreversal of killing. In this report, experiments are described in which the effect of acriflavine on photoreversal of both lethal and mutagenic

damage initiated by UV was determined. Similar experiments in which illumination with visible light preceded exposure to UV (photoprotection)[6, 7] are also described.

Material and Methods.—A tyrosine-requiring derivative of *Escherichia coli* B/r, strain WU36, was used. Cultures were grown overnight, on a shaker, in Difco nutrient broth, with 0.5% NaCl added. One-ml aliquots were diluted in nine ml of fresh nutrient broth, incubated for 45 min, then chilled for ten min in the refrigerator. The bacteria were then washed and resuspended in saline solution (0.9% NaCl) at a titer of about 10^8 cells per ml.

The source of UV was a General Electric germicidal lamp, emitting primarily light of 2537 Å, and having an intensity of 6.6 ergs per second per mm^2 at a distance of 60 cm. The saline suspensions of bacteria were exposed to UV in open Petri dishes, with agitation during the exposure. Yellow light was used to illuminate all subsequent operations, except photoreversal.

The source of photoprotecting or photoreversing light (PPL or PRL) was a 650-watt photoflood bulb in a Westinghouse Studio One camera light. Pre- and post-treatment with visible light was administered just before or after irradiation with UV. The saline suspensions of bacteria were placed in test tubes, which were immersed in a bath of running cold tap water (14°C), at a distance of 3 cm from the lamp. Under these conditions, maximum photoreversal of both killing and induced prototrophy was obtained in strain WU36 after five min of exposure to PRL. "Dark repair" of induced prototrophy does not occur under these conditions, as shown by controls not included here, and can be ignored. Dark controls were treated exactly like the illuminated samples, except that the tubes were wrapped in opaque aluminum foil.

Solid media consisted of minimal "E" medium[8] with 0.4% glucose, supplemented with 5% (by liquid volume) nutrient broth (SEM agar), and SEM to which acriflavine was added, to give a final concentration of 0.0001%, just before pouring the plates (SEM-AC agar). This concentration of acriflavine has no measurable effect on survival or rate of mutation to prototrophy in unirradiated WU36. The bacteria were plated immediately after exposure to PRL in photoreversal experiments, and immediately after irradiation with UV in photoprotection experiments. All incubations were for two days at 37°C. Assays for survival were always performed on the same medium used to determine mutation frequency. Neutral acriflavine was obtained from Nutritional Biochemicals Corporation.

Results.—Figure 1 shows the effects of acriflavine on survival, as a function of UV dose, in the dark, and when visible light is administered before and after exposure to UV. Examining first the dark survival curves, it will be seen that the addition of acriflavine to the postirradiation plating medium markedly decreases UV survival, changing the form of the survival curve to the "one-hit," two-slope type usually obtained with the sensitive B strain. When visible light is administered after UV (PRL curves), the expected photoreversal characteristic of B/r^9 is obtained on SEM plates. About the same amount of photoreversal is obtained on SEM-AC plates, as manifested by the similarity of the "dose reduction" obtained on both kinds of plates. Thus, acriflavine does not significantly interfere with photoreversal of UV killing. The PPL curves show that a small but definite photoprotection against UV killing results from pre-UV exposure to visible light,

whether plating is on SEM or SEM-AC agar. This finding contradicts reports that *E. coli* B/r is not photoprotectible against UV killing.[10, 11] (The wild type B/r strain from which WU36 was derived was found to be equally photoprotectible.) The magnitude of maximal photoprotection is small compared to that of maximal photoreversal, and requires about four times as much visible light. The presence of acriflavine in the postirradiation plating medium does not affect the amount of photoprotection accomplished, as indicated by the similarity of the "dose reduction" on SEM and SEM-AC plates. Acriflavine therefore does not interfere with photoprotection against UV killing.

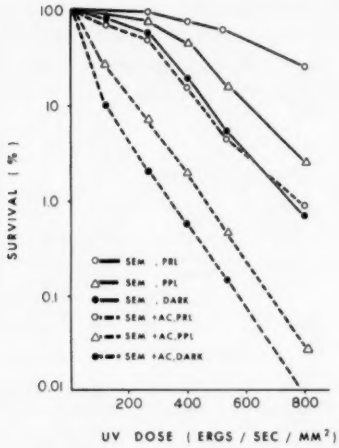

FIG. 1.—Effect of acriflavine on UV survival. Titer at 100% survival = 1.2×10^8 bacteria per ml; PRL—six min of visible light after UV; PPL—20 min of visible light before UV. Each point is the average of three similar experiments.

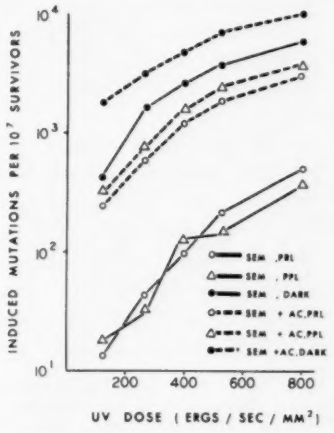

FIG. 2.—Effect of acriflavine on frequency of UV-induced prototrophs. PRL—six min of visible light after UV; PPL—20 min of visible light before UV. Each point is the average of the same three experiments used to determine survival as shown in Fig. 1.

Figure 2 shows the frequency of UV-induced prototrophs, as a function of UV dose, under the same conditions shown in Figure 1 for survival. Examining first the dark controls, it will be seen that somewhat higher yields of induced mutations are obtained on plates containing acriflavine. This "enhancement" effect has been described in an earlier report,[5] and has been interpreted as the consequence of the prevention, by the dye, of residual "dark repair" that normally occurs even on enriched media. The PRL curves show the expected photoreversal of tyr+ mutations on SEM, as described previously.[3] Much less photoreversal of mutation is obtained, however, when plating is on SEM-AC. Acriflavine appears to interfere with photoreversal of these mutations, as well as with their loss by "dark repair." The PPL curves show that pre-UV illumination with visible light reduces the yield of UV-induced prototrophs just as effectively as post-UV illumination with PRL. Photoprotection against induced prototrophy is much more effective than photoprotection against killing, with the same exposure to PPL. When plating is on SEM-AC, relatively little photoprotection against induced prototrophy occurs.

Acriflavine thus interferes with photoprotection against induced prototrophy, as well as with photoreversal and "dark repair" of these mutations.

There are two possible ways of interpreting the smaller degree of photoreversal and photoprotection obtained for induced mutations on SEM-AC plates. The acriflavine may act by affecting the intrinsic reversibility of the premutational lesions leading to induced prototrophy, or it may lower the efficiency of repair so that the amount of visible light used is no longer sufficient to permit maximal repair. To distinguish between these possibilities, experiments were done in which (at a single dose of UV) the time of exposure to PPL or PRL was varied 0–25 min. Results for PRL are shown in Figure 3.

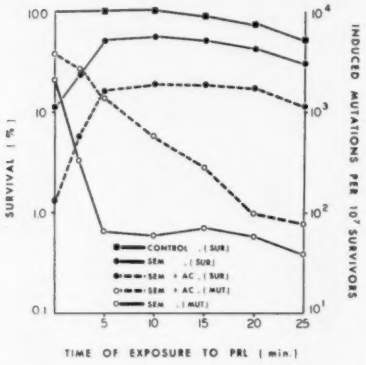

FIG. 3.—Photoreversal of killing and induced prototrophy at various doses of PRL. UV dose—600 ergs per mm;[2] titer at 100% survival = 1.4×10^8 bacteria per ml. Each point is the average of three similar experiments.

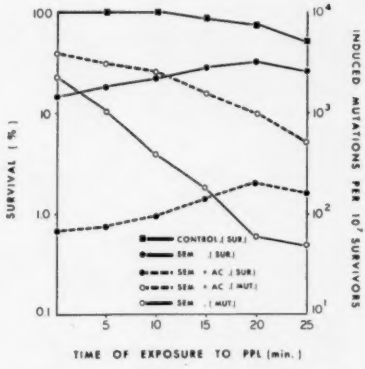

FIG. 4.—Photoprotection against killing and induced prototrophy at various doses of PPL. UV dose—600 ergs per mm;[2] titer at 100% survival = 1.4×10^8 bacteria per ml. Each point is the average of three similar experiments.

The control curve shows the effect of increasing time of exposure to PRL on unirradiated bacteria. Some lethality is observed, starting at about 15 min of exposure. Maximal photoreversal of killing is obtained, on both SEM and SEM-AC plates, with exposures of five min or longer, and the same is true for photoreversal of induced prototrophy when plating is on SEM. When acriflavine is present in the postirradiation plating medium, however, maximal photoreversal of induced prototrophy requires about 20 min of exposure to PRL, as compared with the five min required to accomplish reversal when plating is on SEM. Acriflavine therefore does not alter the *photoreversibility* of the potential mutations, but markedly reduces the *efficiency of the photoreversal*.

A comparable experiment, in which visible light is administered before UV, is shown in Figure 4. Maximal photoprotection against killing requires about 20 min of exposure to PPL, when plating is on SEM or SEM-AC, as does maximal photoprotection against induced prototrophy when plating is on SEM. When plating is on SEM-AC, however, photoprotection against induced prototrophy is still far from the maximal level after 25 min of exposure to PPL. Exposures longer than this are impractical because of lethal effects of PPL itself. It is therefore not possible to determine whether photoprotection would continue, with longer

exposures to PPL, until the achievement of maximal protection. After 20 min of PPL, the same amount of photoprotection is achieved on SEM-AC plates as after five min of PPL on SEM plates. It is clear that the presence of acriflavine in the postirradiation plating medium reduces the efficiency of photoprotection against induced prototrophy.

Discussion.—Differential responses of lethality and mutagenesis to PRL after UV have been noted by Kaplan and Kaplan,[12] working with s-mutations in *Serratia*, from which they concluded that the processes leading to inactivation and mutation are not the same. In other studies,[9, 13, 14] the similarities between photoreversal of the lethal effects of UV and that of the particular mutational systems studied have been more impressive than the differences. The fact that different bacterial mutation systems react differently to UV and PRL, and may originate in different initial steps, was emphasized by Zelle *et al.*[14] There can be little doubt, on the basis of the differential effects of acriflavine, that UV-induced mutations to prototrophy originate through changes which differ from those leading to photoreversible killing.

Considerable recent evidence indicates that photoreversal involves enzymatic repair of UV-produced damage in DNA. A "photoreactivating enzyme," isolated from *E. coli* and yeast,[15] has been shown (when activated by PRL) to mediate the monomerization of dimers of thymine produced by UV in transforming DNA.[16] A mutant strain of *E. coli* B unable to photoreverse UV killing[17] fails to yield "photoreactivating enzyme,"[18] which is readily obtainable from the photoreactivable parent strain. This strongly suggests (unless one assumes more than one specific activity for this enzyme) that most, if not all, of the photoreversible killing in *E. coli* is the consequence of dimerization of thymine. This possibility is consistent with the finding[19] that thymine dimers account for about half the lethal effect of high doses of UV.

Acriflavine interferes with photoreversal of induced prototrophy, but not with photoreversal of killing. Assuming both kinds of photoreversal to be enzymatic, and further assuming that photoreversible killing is largely initiated by dimerization of thymine, there are two possible interpretations of this result. The simpler would be that two distinct enzymes are involved, each acting on a different primary lesion. In this case, it would follow that mutations to prototrophy do not originate through thymine dimers, but through one or more other kinds of UV-initiated lesion in DNA.

It is also possible, however, that the same "photoreactivating enzyme" is involved in the repair of lesions responsible for photoreversible killing and induced prototrophy. If this is so, one must suppose that the initial lesions leading to death and to prototrophy are the same (presumably thymine dimers), and that the differentiating effect of acriflavine depends upon a secondary difference. For example, one might imagine that thymine dimers cross-linking the two strands of DNA (if they occur) and those involving neighboring bases in the same strand could be monomerized by the same enzyme, yet they might be differentially affected by the combination of acriflavine with the DNA. This, or some other secondary difference in spatial organization could result in differential availability of prelethal and preprototrophic lesions for repair by the same enzyme. The fact that induced prototrophy is subject to "dark repair," while UV killing is not, raises the same question. This difference, too, could be explained by assuming that different primary lesions

are involved, or that the difference is in the accessibility of the lesions to the reparative action of the postulated "dark repair" enzyme. Decisive answers are being sought in studies, now in progress, of mutant strains lacking one or another of the repair enzymes.

Summary.—The addition of acriflavine to the postirradiation plating medium has the following effects in strain WU36, a tyrosine-requiring substrain of *E. coli* B/r: (1) it "enhances" both the lethal and mutagenic effects of UV, decreasing survival and increasing the yield of tyr+ mutations; (2) it reduces the efficiency of photoreversal of induced prototrophs, increasing the amount of light required to accomplish maximal photoreversal about fourfold, while having no such effect on the photoreversal of UV killing; (3) it reduces the efficiency of photoprotection against induced prototrophy, while having no such effect on photoprotection against UV killing. Mutations to prototrophy are reducible in frequency by pretreatment with visible light to the same extent as by posttreatment with visible light, although maximal photoprotection requires about four times as much light as maximal photoreversal. In contrast, very little photoprotection against UV killing is obtained in this strain. The results are interpreted as evidence that the lesions leading to photoreversible UV killing and those leading to photoreversible UV-induced prototrophy are different, either in their primary nature or in some unknown secondary feature.

These studies were carried out with the able technical assistance of Mr. Nicholas A. Sicurella.

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*SIMPLIFICATION OF MAPPINGS**

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Communicated July 15, 1963

1. *Introduction.* In this paper there is developed a factorization process for mappings which can be used to draw together singularities of certain light open mappings so as to exhibit their action in the large in terms of classical power mappings. All spaces used will be assumed separable and metric and a *mapping* $f(X) = Y$ is assumed continuous. Such an f is *open* (closed) provided the image of every open (closed) set in X is open (closed) in Y, and is light provided the inverse $f^{-1}(y)$ of each point in Y is totally disconnected.

If $f(X) = Y$ is a mapping, an *inverse set* X_0 is a set in X satisfying $X_0 = f^{-1}f(X_0)$. The mapping is *quasi-compact* provided the image of every open inverse set in X is open in Y. It may be recalled that the natural mapping of a decomposition of X is always quasi-compact.

2. *General Factorization Theorem.* We will be concerned with the situation in which the domain and range spaces X and Y of a mapping f are decomposed, yielding quotient spaces X' and Y'; and we develop conditions under which a useful associated mapping g can be introduced in terms of f and the natural mappings of these decompositions. To this end, consider mappings

$$f(X) = Y,\ \phi_1(X) = X', \text{ and } \phi_2(Y) = Y',$$

where it is assumed that ϕ_1 and ϕ_2 are quasi-compact. We impose the additional conditions:

(a) *For each* $x' \in X'$, $f\phi_1^{-1}(x') = \phi_2^{-1}(y')$ *for some* $y' \in Y'$,

(b) *For each* $y' \in Y'$, $f^{-1}\phi_2^{-1}(y') = \phi_1^{-1}(T)$ *for some set* $T \subset X'$, and define $g(x') = \phi_2 f \phi_1^{-1}(x')$ for $x' \in X'$.

(2.1) Theorem. *Under these conditions,* $g = \phi_2 f \phi_1^{-1}$ *is single-valued and continuous, maps* X' *onto* Y', *and is open* (closed) *when* f *is open* (closed).

Condition (a) makes g single-valued. To show continuity, let U be any open set in Y'. By condition (b), $f^{-1}\phi_2^{-1}(U)$ is an inverse set for ϕ_1; and this set is open by continuity of ϕ_2 and f. Thus, by quasi-compactness of ϕ_1, $\phi_1 f^{-1}\phi_2^{-1}(U)$ is open in X'; and since this set is identical with $g^{-1}(U)$, g is continuous.

Now suppose f is open, and let V be any open set in X'. Then $f\phi_1^{-1}(V)$ is open by continuity of ϕ_1 and openness of f. Also, this set is an inverse set for ϕ_2 by condition (a). Thus, $g(V) = \phi_2 f \phi_1^{-1}(V)$ is open by quasi-compactness of ϕ_2.

(2.11) Corollary. *If for a set* $G' \subset Y'$, ϕ_2 *is* $1 - 1$ *on* $Y' - G'$, *and* ϕ_1 *is the natural mapping of the decomposition of* X *into the sets* $f^{-1}\phi_2^{-1}(y')$, $y' \in G'$, *and single points of the rest of* X, *conditions* (a) *and* (b) *are satisfied. Further,* g *is light if* f *is light and* ϕ_1 *is monotone.*

To check the last statement here, we note that if P is a nondegenerate connected set in X', $f\phi_1^{-1}(P) = Q$ is nondegenerate and connected by monotoneity of ϕ_1 and lightness of f. Hence, $\phi_2(Q)$, which is $g(P)$, cannot be a single point y', because we would have $y' \in G'$ so that $\phi_1 f^{-1}\phi_2^{-1}(y')$ would be a single point of X' whereas it contains P.

Of particular interest to us here will be the case in which G' consists of a single point or a pair of points or, equivalently, where ϕ_2 is generated by decomposing Y into a single closed set or two disjoint closed sets plus single points.

(2.12) COROLLARY. *Let X and Y be locally compact and let Y_0 be a closed set in Y such that all components of both Y_0 and $X_0 = f^{-1}(Y_0)$ are compact. Let ϕ_1 and ϕ_2 be the natural mappings of the decompositions of X and Y into the components of X_0 and Y_0, respectively, and single points of their complements. Then if f is open, conditions* (a) *and* (b) *are satisfied so that g is open. Also, g is light if f is light.*

For condition (a), let $x' \in X'$, and assume $x' \in \phi_1(X_0)$ as otherwise (a) follows trivially. Then $\phi_1^{-1}(x')$ is a component of X_0. Hence, $f\phi_1^{-1}(x')$ is a component of Y_0 by openness of f and local compactness of X. Thus, this set is the ϕ_2 inverse of some point of Y'. For (b), if $y' \in Y'$, $\phi_2^{-1}(y')$ is a component of Y_0. Thus, $f^{-1}\phi_2^{-1}(y')$ is the union of a set of components of X_0 and hence is the ϕ_1 inverse of some set T in X'. Lightness of g follows about as in the preceding corollary, since now both ϕ_1 and ϕ_2 are monotone.

3. *Examples. 2-cell mappings:* As an illustration of how the $\phi_2 f \phi_1^{-1}$ process serves to generate mappings, let f be the power mapping $w = z^k$ of the complex z-sphere X onto the w-sphere Y. Then if we choose any single point p other than o and ∞ on Y, let ϕ_2 be the identity mapping, and decompose X into the set $f^{-1}(p)$ and single points as in (2.1); letting $\phi_1(X) = X'$ be the natural mapping of this decomposition, we get for g a light, open mapping of degree k of the pseudomanifold X' (of genus $k - 1$) onto the sphere Y.

To see how this process may be used in simplification of a mapping, let f be the mapping $w = z^4 - z^3$ of X onto Y (complex spheres). Here there are singular points on Y at 0, $-27/256 = c$ and ∞, and these have inverses $[0,1]$, $[3/4, -1/4 \pm 1/4\ \sqrt{2}\ i]$, and ∞, respectively. Let Y_0 be the interval joining c and 0. It can then be shown, and indeed it follows from results below, that the set $X_0 = f^{-1}(Y_0)$ is a continuum not separating X. Thus, if we apply the $\phi_2 f \phi_1^{-1}$ process as outlined in (2.12), all conditions there are satisfied. Further, X' and Y' are topological spheres and the mapping g thus generated is topologically equivalent to $w = z^4$. In the next section, conditions will be developed under which an arbitrary light, open mapping of a 2-sphere onto a 2-sphere can thus be reduced to a power mapping.

Definition: If A and B are 2-manifolds, a mapping $f(A) = B$ is *normal*, provided the edge set of A is precisely the full inverse of the edge set of B. Thus, edge points map into edge points, and ordinary points into ordinary points. If A and B are compact, it will be recalled that for a light, open, normal mapping $f(A) = B$ we have the basic relation

$$k\chi(B) - \chi(A) = kr - n$$

connecting the degree k of f, the numbers r of singular points on B and n of their inverse points on A, and the Euler characteristics $\chi(A)$ and $\chi(B)$.

A closed subset K of a continuum M is *pointlike* in M provided $M - K$ and $M - a$ are homeomorphic for some point a in M. It is readily verified from well-known results that for any compact 2-manifold M contained in a 2-sphere S^2, a continuum $K \subset \text{int. } M$ is pointlike in M if and only if $M - K$ is connected.

Note: For a subset Q of a space X, *int.* Q denotes the interior of Q relative to

X; but in case Q is a manifold, int. Q means the set of all ordinary (i.e., nonedge) points of Q.

(3.1) THEOREM. *Let $f(A) = B$ be light, open, and normal where A is a 2-manifold contained in S^2, and B is a 2-cell. Let β be any continuum in int. B containing all singular points of f and not separating B, and let $\alpha = f^{-1}(\beta)$. Then A is a 2-cell if and only if α and $A - \alpha$ are both connected.*

Proof: Suppose first that *some such* β exists so that α and $A - \alpha$ are connected. Then since α is pointlike in A, $A' = \phi_1(A)$ is homeomorphic with A, where ϕ_1 is the natural mapping of the decomposition of A into α and single points of $A - \alpha$. Defining $\phi_2(B) = B'$ similarly, for the light, open, normal mapping $g = \phi_2 f \phi_1^{-1}$ of A' onto B' we have

$$\chi(B') = \chi(B) = 1 \text{ and } r = 1,$$

so that

$$k - \chi(A') = k - n = k - \chi(A);$$

whence $\chi(A) = 1$, so that A is necessarily a 2-cell.

On the other hand, let A be a 2-cell and let β be *any continuum in int. B* satisfying the conditions stated. Then $A - \alpha$ must be connected because, by openness of f, each of its components maps onto $B - \beta$ and thus contains the edge of A. Hence, no component of α separates A, and if $\phi_1(A) = A'$ is the natural mapping of the decomposition of A into components of α and single points of $A - \alpha$ as in (2.12), A' is a 2-cell. For the mapping $g = \phi_2 f \phi_1^{-1}$ of A' onto B', where $\phi_2(B) = B'$ is similarly defined, we have $k - 1 = k - n$, so that $n = 1$ and α is connected.

(3.11) COROLLARY. *With A, B, and f as in* (3.1), *if for some pointlike set $B \subset$ int. B containing all singular points of f on B, $f^{-1}(\beta)$ is pointlike, then A is a 2-cell and the corresponding mapping $g = \phi_2 f \phi_1^{-1}$ is equivalent to a power mapping.*

(3.12) COROLLARY. *If $f(A) = B$ is light, open, and normal where A and B are 2-cells, then for any pointlike set $\beta \subset$ int. B containing all singular points of f on B, $f^{-1}(\beta)$ is pointlike.*

4. *Polynomial Type Mappings.* If X and Y are 2-spheres, a light open mapping $f(X) = Y$ of degree k is of *polynomial type*, provided there exist monotone mappings $\phi_1(X) = X'$ and $\phi_2(Y) = Y'$ with X' and Y' 2-spheres and satisfying conditions (a) and (b) of section 2 and such that the mapping $g = \phi_2 f \phi_1^{-1}$ of X' onto Y' is topologically equivalent to a power mapping of degree k. It is a consequence of the theorem to be proved below that the mapping generated by any complex polynomial or the reciprocal of a polynomial is of this type, and hence is reducible to a power mapping by the $\phi_2 f \phi^{-1}$ process with ϕ_1 and ϕ_2 monotone. However, not all rational functions are of polynomial type.

(4.1) THEOREM. *If X and Y are 2-spheres, in order that a light, open mapping $f(X) = Y$ be of polynomial type, it is necessary and sufficient that there exist a continuum β in Y not separating Y whose inverse α is connected and does not separate X.*

Suppose first that such a continuum β exists in Y. Let β_1 be a simple arc in $Y - \beta$ containing all singular points of f in Y which are not on β. Let C be a simple closed curve in $Y - \beta - \beta_1$ separating β and β_1 in Y and thus decomposing Y into two 2-cells B and B_1 meeting in their common edge C and containing β and β_1, respectively. Let $A = f^{-1}(B)$ and $A_1 = f^{-1}(B_1)$. Then since A is a 2-

manifold and the mapping $f|A$ is light, open, and normal while β, $B - \beta$, α, and $A - \alpha$ are all connected, it follows by (3.1) that A is a 2-cell.

Now since $A + A_1 = X$, it follows that A_1 also is a 2-cell. Since β_1 and $B_1 - \beta_1$ are connected and β_1 contains all the singular points of $f|A_1$ on B_1, again it follows from (3.1) that if $\alpha_1 = f^{-1}(\beta_1)$, then both α_1 and $A_1 - \alpha_1$ are connected. Thus, if $\phi_1(X) = X'$ and $\phi_2(Y) = Y'$ are natural mappings for the decompositions of X into α, α_1, and points, and of Y into β, β_1, and points, respectively, both X' and Y' are 2-spheres; and since $\phi_1(\alpha)$ and $\phi_1(\alpha_1)$ are the only singular points for the mapping $g = \phi_2 f \phi_1^{-1}$, it is clear that g is topologically equivalent to the power mapping $w = z^k$ where k is the degree of f.

To prove the necessity of the condition, let $g = \phi_2 f \phi_1^{-1}$ be equivalent to $w = z^k$ where X, X', Y, Y' are 2-spheres, $\phi_1(X) = X'$ and $\phi_2(Y) = Y'$ are monotone, $f(X) = Y$ is light and open and of degree k, and conditions (a) and (b) of section 2 hold. Let O and $O' = g(O)$ be the points on X' and Y' corresponding to the origins in the z and w planes, respectively. Then if we define $\alpha = \phi_1^{-1}(O)$, $\beta = f\phi_1^{-1}(O) = \phi_2^{-1}(O')$, we have $\alpha = f^{-1}(\beta)$. Also α and $X - \alpha$ are connected by monotoneity of ϕ_1; and β and $Y - \beta$ are connected by monotoneity of ϕ_2.

(4.11) COROLLARY. *If there is a point in Y with a unique inverse point under f, then f is of polynomial type. (Thus, the mapping generated by any polynomial is of polynomial type.)*

(4.12) COROLLARY. *A mapping f as in* (4.1) *is of polynomial type if and only if Y contains a simple arc (or a pointlike set) β whose inverse set is pointlike.*

In the proof of (4.1) we have also proved:

(4.2) THEOREM. *Let $f(X) = Y$ be light and open where X and Y are 2-spheres. If Q is a pointlike set in Y with a pointlike inverse, any pointlike set in Y containing all singular points of f not on Q has a pointlike inverse.*

Since any single point in Y is pointlike but can have a pointlike inverse only when its inverse is a single point, we have: (*i*) if there is a point q in Y with a unique inverse, any arc in $Y - q$ containing all singular points of f other than q has a pointlike inverse, and (*ii*) if there is an arc β in Y containing all singular points except one, say q, and having a pointlike inverse, then q has a unique inverse. Hence,

(4.21) *There is a point $q \in Y$ with a unique inverse if and only if there exists a pointlike set (or simple arc) in Y containing all save at most one of the singular points of f and having a pointlike inverse.*

If an arc exists in Y containing *all* singular points of f and having a pointlike inverse, all points not on this arc have unique inverses and f is a homeomorphism. Thus, such an arc can never exist when there are singular points.

It follows from the above that any f, from 2-sphere to 2-sphere, for which a point with a unique inverse exists, is of polynomial type. A mapping of polynomial type need not admit such a point, however. For example, the mapping defined by $w = z^4 - z^3$ on $|z| \leq 2$ and defined by duplication of this action on the complementary 2-cell of the complex z-sphere would be of polynomial type but admits no point with a unique universe. Such a mapping would be given by the rational function $w = z^4 - z^3/z - 2$.

Not all rational functions generate mappings of polynomial type, e.g., $w = (1 + z^2/1 - z^2)^2$. Here, in effect, we have a square mapping rotating the sphere

about a North-South axis followed by a similar rotation about an East-West axis. This mapping cannot be reduced to a simpler type by the $\phi_2 f \phi_1^{-1}$ process.

It would be interesting to identify, algebraically or analytically, the classes of functions corresponding to the polynomial, reducible, and irreducible types of mappings as discussed here.

5. *Reduction of 2-Manifold Mappings.* It will be recalled that every compact 2-manifold M without boundary is constructible by identifying selected edge pairings on a simple polygon. Thus, every such manifold M contains at least one finite connected graph β, namely, the image of the edge of the basic polygon, which cuts M into an open 2-cell.

(5.1) THEOREM. *Let $f(X) = Y$ be light and open where X and Y are compact 2-manifolds without boundary, and let β be a finite connected graph on Y such that $Y - \beta$ is an open 2-cell and so that β either* (1) *contains all singular points of f on Y, or* (2) *contains all save one of these singular points. Then $\alpha = f^{-1}(\beta)$ is connected in either case. Further, in case* (1) *each component of $X - \alpha$ maps topologically onto $Y - \beta$, while in case* (2) *each component of $X - \alpha$ maps onto $Y - \beta$ by a power mapping. Thus, if q is the singular point of f in $Y - \beta$, each component of $X - \alpha$ contains just one point of $f^{-1}(q)$.*

Proof: Let B be any closed 2-cell in $Y - \beta$ which, in case (2), contains the singular point in its interior; and let A be any component of $f^{-1}(B)$. Since A is a 2-manifold and the mapping $f(A) = B$ is light, open, and normal and of degree k_1, we have the relation

$$\begin{aligned} k_1 - \chi(A) &= k_1 - n_1, \text{ in case (2)} \\ &= 0, \text{ in case (1), since } \chi(B) = 1. \end{aligned}$$

Thus, $\chi(A) = k_1$, in case (1), and $\chi(A) = n_1$ in case (2). In either case, it follows that $\chi(A) = 1$ and A is a 2-cell, since only the 2-cell has edge points and a positive characteristic. Hence, in case (1) $f(A) = B$ is topological, while in case (2) $n_1 = 1$ so that $f(A) = B$ is a power mapping.

Since B can be taken "arbitrarily near" $Y - \beta$, i.e., so as to contain any preassigned compact subset of $Y - \beta$, all our conclusions except the connectedness of α follow. To see that this latter also holds, we note that if J is the edge of B, then $C = f^{-1}(J) \cdot A$ is the edge of A. Thus, the boundary of the component Q of $X - \alpha$ containing A must be connected, as it is the limit of a sequence of simple closed curves lying in Q. It follows from this and local connectedness of X that α is connected.

Now suppose we apply the $\phi_2 f \phi_1^{-1}$ process by letting the mappings $\phi_1(X) = X'$ and $\phi_2(Y) = Y'$ be generated by decomposing X into α plus single points and Y into β plus single points, respectively. It is clear then that we have

(5.11). COROLLARY. *The quotient spaces X' and Y' are, respectively, a finite cluster of 2-spheres and a single 2-sphere. The associated mapping $g = \phi_2 f \phi_1^{-1}$ of X' onto Y' maps each sphere of the cluster forming X' onto the sphere Y' topologically in case* (1) *and by a power mapping in case* (2).

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*EFFECTS OF PUROMYCIN ON RNA SYNTHESIS IN MAMMALIAN CELLS**

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Puromycin is a potent inhibitor of protein synthesis both *in vitro* and *in vivo*.[1, 2] In addition, it is known to inhibit RNA replication of several RNA-containing mammalian viruses.[3–5] In studying this effect on poliovirus RNA synthesis, we have observed a depression of RNA synthesis in control uninfected HeLa cells. This seemed to differ from findings in bacteria where chloramphenicol inhibition of protein synthesis allows continued synthesis of bacterial ribosomal and transfer RNA[6, 7] as well as phage messenger RNA.[8]

The present report shows that puromycin inhibits synthesis of 30S and 18S ribosomal RNA and of larger ribosomal precursor RNA. Under the same conditions, puromycin is less inhibitory to the synthesis of transfer RNA and to the synthesis of an unstable RNA which may be mammalian cell messenger RNA. After this work was completed, a similar finding of puromycin inhibition of ribosomal RNA synthesis was published by Tamaoki and Mueller.[9] The present paper provides inadvertent confirmation of their findings and, at the same time, further documentation and extension of their findings and conclusions.

Materials and Methods.—HeLa cell monolayers were grown in a medium containing 7% calf serum and 0.1% yeast extract and 0.1% Difco-proteose peptone in Hanks' balanced salt solution. Cells were kept in logarithmic phase of growth by frequent change of medium.

All experiments were carried out using Eagle's medium with or without addition of inhibitors and P^{32}. P^{32} labeling was accomplished by incubation of about 10^7 cells (treated or not as indicated) in Eagle's medium containing 5% dialyzed calf serum and 100 μc/ml of P^{32} orthophosphate as the sole added source of phosphorus.

After incubation for 3.5 hr at 37°, excess P^{32} was removed by washing with 0.15 *M* NaCl, and RNA was extracted with phenol. The cells were shaken with equal volumes of water-saturated phenol and 0.02 *M* pH 7.2 phosphate buffer at room temperature. After centrifugation, the aqueous phase containing ribosomal RNA and transfer RNA was removed, the phenol phase was discarded, and the interphase nucleoprotein precipitate was re-extracted twice with phenol-buffer.

Phenol extraction under the above conditions of pH and ionic strength results in fractionation of mammalian cell RNA into 2 classes:[10–12] the aqueous phase RNA (pRNA) containing about 90% of total cellular RNA; and the residual interphase RNA (rRNA) associated with DNA and protein precipitated at the interphase (comprising about 10% of total cellular RNA).[13]

Aqueous phase pRNA was precipitated several times with ethanol, then was freed of bound contaminating radioactive compounds by 3 precipitations from 2 *M* potassium acetate solution, brought to 30% ethanol and chilled to −20° for 2 hr to allow precipitation.[14] Precipitation in the cold from potassium acetate has proved to be the best method tested for removal of radioactive contaminants without breakdown of the RNA detectable by sedimentation analysis. Failure to remove tightly bound radioactivity leads to inaccurate determinations of RNA synthetic rates and sucrose sedimentation patterns, but precipitation from potassium acetate yielded RNA in which almost all radioactivity could be recovered in the separated 2′, 3′ nucleotides after alkaline hydrolysis.

After removing the aqueous phase and discarding the phenol phase, the nucleoprotein interphase precipitate was twice more extracted with phenol-buffer, and the nucleoprotein was washed and precipitated 3 times from 95% ethanol. DNA and rRNA were then extracted from the precipitate by adding 1.5 ml of 5% sodium lauryl sulfate solution containing 0.1 *M* citrate and 0.001 *M* EDTA, stirring for 3 min at 60°, diluting with 5 ml 0.15 *M* NaCl, and stirring 3 additional min

at 60°. Finally, an equal volume of water-saturated phenol was added, and the mixture shaken vigorously for 3 min at 60°, then centrifuged, and the aqueous phase containing DNA and rRNA was removed by pipetting. DNA and rRNA were then precipitated with ethanol and reprecipitated 3 times from 2 *M* potassium acetate as described above for pRNA. Where only radioactivity was to be measured, carrier ribosomal RNA was added to facilitate precipitation and manipulation. Sodium lauryl sulfate treatment never released all nucleic acid from the denatured nucleoprotein interphase precipitate; but if phenol extractions were completed quickly so that 30 min or less elapsed between the first phenol treatment and addition of sodium lauryl sulfate, maximal release of DNA and rRNA was obtained.

Sucrose gradient sedimentation analysis[15] of pRNA and rRNA was performed using the Spinco SW 39 swinging bucket head. All runs were carried out in a 5–20% linear sucrose gradient containing 0.001 *M* EDTA, 0.1 *M* NaCl, and 0.01 *M* tris-HCl buffer, pH 7.2. RNA was dissolved in 0.15 *M* NaCl and carefully layered over 4.7 ml sucrose gradients in 0.3 ml amounts. After centrifugation at 38,000 rpm for 6 hr, 2 drop fractions were collected from the bottom of the tube and were analyzed for radioactivity and optical density and, in some cases, pooled fractions were degraded to nucleotides and base composition determined.

Base ratios were determined after electrophoretic separation of nucleotides obtained by alkaline hydrolysis of RNA. Electrophoretic separation was carried out in pH 3.5 citrate buffer using 1,200 volts for 6 hr (16 volts/cm).[16, 17] Radioactivity of each nucleotide was determined with a Vanguard strip scanner with integrating data system.

Aggregate enzyme RNA polymerase from HeLa cell nuclei was prepared by a modification of the method of Weiss[18] and assayed in the high ionic strength reaction mixture of Goldberg[19] using ATP labeled in the alpha position with P^{32}. Details of the method and preparation of ATP^{32} P.P. have been described previously.[20] Product RNA was isolated by extraction with phenol-sodium lauryl sulfate and 3X precipitated from 2 *M* potassium acetate as above, then heated at 100° for 30 sec to release nascent RNA annealed to DNA.

Puromycin was generously provided by Lederle Laboratories. It was added to the cell culture medium or labeling medium at a concentration of 100 μg/ml, a level which inhibited over 95% of HeLa protein synthesis within 2 hr.

Results.—Effect of puromycin on ribosomal RNA synthesis: Comparison of pRNA synthesis or rRNA synthesis in normal HeLa cells and puromycin-treated HeLa cells consistently showed that puromycin reduced the rate of synthesis of RNA, but never completely suppressed synthesis (Table 1). When pRNA was

TABLE 1

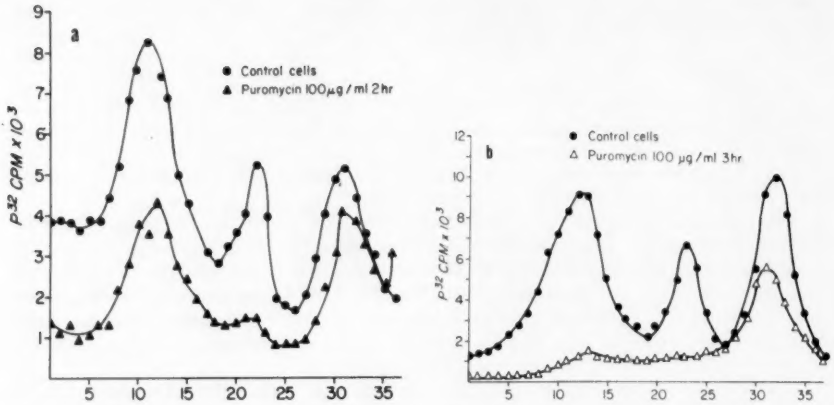
EFFECT OF PUROMYCIN ON pRNA AND rRNA SYNTHESIS

Experiment	Hr exposure to puromycin* (100 μg/ml)	Per cent P^{32} incorporated into RNA after puromycin treatment as compared to untreated control cells†	
		pRNA	rRNA
1	6.5	15	18
2	6	35	40
3	4	29	40
4	4	28	46
5	4	41	21
6	3	36	51
7	2	31	37
8	1	67	73

* Cells were treated with puromycin (100 μg/ml) for the times indicated immediately before extracting nucleic acid.

† In each experiment P^{32} labeling was carried out for 3.5 hr prior to nucleic acid extraction regardless of the presence or absence of puromycin.

analyzed by sedimentation in a sucrose gradient (Fig. 1), it was found that puromycin treatment led to inhibition of 30S and 18S ribosomal RNA synthesis, while 4–8S soluble RNA continued to be synthesized at near normal rates. The magnitude of inhibition of 30S RNA varied in different experiments, but there was consistent inhibition of ribosomal RNA synthesis and only slight inhibition of soluble



FIGS. 1*a* and *b*.—Two different experiments showing varying degrees of inhibition of ribosomal RNA synthesis by treatment of HeLa cells with 100 μg/ml puromycin for several hr. P^{32}-labeled RNA extracted into the aqueous phase by phenol treatment (pRNA) was layered over 5–20% sucrose linear gradients and centrifuged for 6 hr at 38,000 rpm in the Spinco SW 39 swinging bucket rotor. The sucrose solutions used to establish the gradient were buffered to pH 7.2 with tris-HCl buffer (0.01 *M*) and also contained 0.1 *M* NaCl and 0.001 *M* EDTA.

RNA synthesis. Prolonged treatment with puromycin usually caused more complete inhibition of ribosomal RNA synthesis than short treatment, although in some experiments exposure to puromycin for 2 hr showed marked inhibition.

Puromycin treatment had no effect on total ribosomal RNA measured spectrophotometrically, so it apparently acts by inhibiting ribosomal RNA synthesis rather than by breaking down already formed ribosomal RNA. This was confirmed by the finding that puromycin treatment of cells, labeled with P^{32} 24 hr previously, affected neither the amount of P^{32} in pRNA nor the sedimentation rate or quantity of P^{32} in 30S or 18S ribosomal RNA.

Effect of puromycin on ribosomal precursor RNA and other rRNA: It has been demonstrated by Georgiev and co-workers,[10] and by Sibatani and colleagues,[11, 12] that phenol nonextractable (r) RNA differs from the bulk of mammalian cell RNA in having a higher turnover rate and a different base composition. Hiatt[14] showed that pretreatment of liver cells with sodium dodecyl sulfate was necessary to enable extraction of this rapidly labeled RNA into the aqueous phase during phenol treatment. Scherrer *et al.*[21] have recently shown that sodium dodecyl sulfate treatment before phenol extraction of HeLa cells allows isolation of this material as large (35S and 45S) rapidly labeled RNA which appears to be ribosomal precursor RNA. We demonstrated earlier that HeLa cell rRNA differs markedly from bulk ribosomal and soluble pRNA in base composition.[13] Figure 2 shows a sucrose sedimentation diagram of P^{32}-labeled rRNA released from a phenol interphase precipitate by sodium lauryl sulfate treatment. Radioactivity is found throughout the tube representing molecules from 50S in size to molecules smaller than 6S. Because of the difficulty in extracting rRNA from denatured protein, precipitates without appreciable breakdown sedimentation profiles differ in each run, but in every case RNA ranging in size from very large to small molecules is found. DNA is also present in these extracts but contributes negligible radioactivity under these con-

ditions of labeling. All extracts are heated at 100° for 30 sec and quickly cooled before sucrose sedimentation to avoid cosedimentation of rRNA annealed to DNA. Optical density of added HeLa cell 30S and 18S ribosomal RNA and 4–85 transfer RNA are included as references.

Figure 2 also shows base ratios of pooled rRNA fractions from 3 portions of the sucrose gradient. It can be seen that the largest rRNA molecules have high guanine-cytosine base compositions corresponding to those of ribosomal RNA. This appears to be the large precursor of ribosomal RNA detected after pulse-labeling by Scherrer *et al.*[21] However, the more slowly sedimenting rRNA molecules (up to 20S) exhibit high uracil base ratios which are not inconsistent with their being messenger RNA. As reported previously,[13] the total rRNA obtained by direct alkaline hydrolysis of the phenol interphase precipitate is also high in uracil, but this obviously includes ribosomal precursor RNA having the high G-C base ratios characteristic of ribosomal RNA.

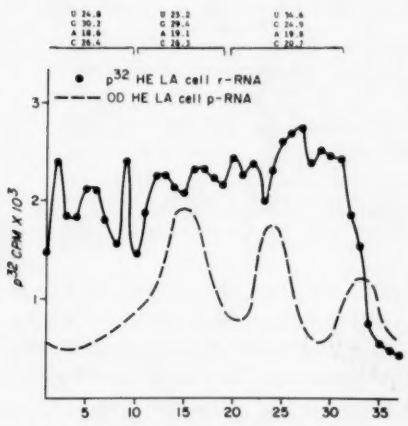


FIG. 2.—Sucrose sedimentation diagram of P^{32}-labeled rRNA extracted by detergent from the interphase precipitate of phenol-treated HeLa cells. Sedimentation conditions as for Fig. 1, except that rRNA was heated at 100°C for 30 sec and quickly cooled before sucrose sedimentation runs (to prevent cosedimentation of RNA annealed to DNA).

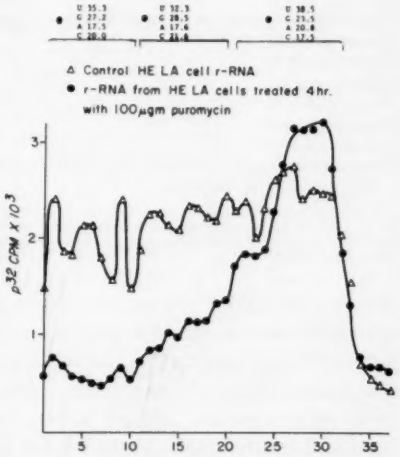


FIG. 3.—Sucrose sedimentation diagram comparing the sedimentation of rRNA from normal HeLa cells (see Fig. 2) and rRNA from cells treated 3 hr with 100 μg/ml puromycin. The base ratios of RNA from pooled fractions of rRNA of puromycin-treated cells are shown.

Figure 3 shows a sucrose sedimentation pattern exhibited by rRNA extracted from normal cells and an aliquot of the same cells treated 4 hr with 100 μgm/ml puromycin. Puromycin treatment inhibited synthesis of rapidly sedimenting ribosomal precursor RNA, leaving a broad distribution of RNA having a peak 10–20S and exhibiting very high uracil base ratios at all levels of the sucrose gradient.

Thus, puromycin arrested synthesis of ribosomal RNA and ribosomal precursor RNA, and allowed synthesis of a high uracil RNA which might be messenger RNA. The stability of this RNA was examined by labeling cells with P^{32} for 4.0 hr, then removing P^{32} medium and incubating in medium containing cold inorganic phosphate for 20 hr before extracting pRNA and rRNA. It was found that almost

all label was in ribosomal and soluble RNA of the pRNA fraction and in DNA. No ribosomal precursor rRNA was detectable in sucrose gradients, and only about 10 per cent of the high uracil rRNA present 20 hr previously could be found in the 10–20S region of the gradient. This rRNA had a base composition differing (U = 32.2, G = 34.4, A = 16.0, C = 17.4) from that found before the 20 hr chase. Whether this is a population of RNA molecules that may include "stable messenger" is speculative, but it is clear that the bulk of rRNA (including both ribosomal precursor RNA and the smaller high uracil base ratio molecules) turns over within one generation time (generation time = 20–30 hr).

RNA polymerase activity of HeLa cells treated with puromycin: Since puromycin arrests ribosomal RNA synthesis of intact cells, it was of interest to determine its effect on cellular RNA polymerase activity. Table 2 shows that exposure of HeLa

TABLE 2

EFFECT OF PUROMYCIN ON HELA CELL AGGREGATE ENZYME RNA POLYMERASE ACTIVITY

Treatment of cells before extraction of aggregate enzyme	ATP^{32} incorporated into RNA (mμm/mg)*	Per cent of control incorporation
None (control)	0.44	100
Puromycin 6 hr	0.35	80
None (control)	0.38	100
Puromycin 6 hr	0.34	89
None (control)	0.49	100
Puromycin 6 hr	0.35	71
None, but 100 μg/ml puromycin added to aggregate enzyme reaction mixture	0.47	96

* The RNA polymerase reaction mixture (modified from Weiss[18] and Goldberg[19]) contained in a total volume of 0.5 ml: 50 μM tris buffer, pH 8.0; 0.4 μM UTP, CTP, GTP; 5 μM B mercaptoethanol; 1.5 μM $MnCl_2$; 0.05 ml of saturated $(NH_4)_2SO_4$; 0.4 μM alpha-labeled ATP^{32}; and about 1 mg of "aggregate enzyme" protein with associated DNA.

cells to 100 μg/ml puromycin results in loss of less than 30 per cent of original aggregate enzyme RNA polymerase activity within 6 hr. Figure 4 shows a sucrose sedimentation diagram of the RNA produced by aggregate enzyme from normal and treated cells. In each case, a broad distribution of RNA is observed with a peak at about 15S. Either a very small proportion of ribosomal size RNA is produced by this DNA-primed RNA polymerase or it is degraded to smaller fragments during incubation in the reaction mixture. It is likely that most of the RNA produced by this DNA-enzyme aggregate is equivalent to the informational RNA of the intact cells. It is interesting therefore that it is similar in size to the high uracil rRNA synthesized by intact cells, which also may contain informational RNA. The amount of RNA polymerase activity surviving 6 hr inhibition of protein synthesis by puromycin indicates that aggregate enzyme is rather stable and does not turn over rapidly. However, the enzyme that polymerizes ribosomal RNA might turn over rapidly, and this might then contribute to the small decrease in aggregate enzyme that does result from puromycin treatment. Puromycin added to the RNA polymerase reaction mixture had no effect on *in vitro* RNA synthesis (Table 2).

Discussion.—It seems reasonable to assume that, whatever the mechanism of puromycin depression of ribosomal RNA synthesis may be, it is a secondary effect resulting from inhibition of protein synthesis by puromycin. It is possible, but not likely, that interruption of protein synthesis leads to repression of the chromosomal locus controlling ribosomal RNA synthesis. In *Escherichia coli* it appears

that deprivation of an essential amino acid leads to repression of the ribosomal locus.[22, 7] However, in *E. coli*, interruption of protein synthesis by other means (by chloramphenicol) does not cause immediate repression of ribosomal RNA synthesis.[6, 7] Chloramphenicol treatment prevents normal ribosome synthesis and leads to production of "CM particles" containing ribosomal RNA associated with a small amount of protein.[23] Rather than inhibiting RNA synthesis, chloramphenicol may greatly stimulate ribosomal RNA incorporation into "CM particles" in bacteria grown in minimal medium.[7]

As mentioned above, the RNA polymerase for ribosomal RNA may turn over rapidly and therefore require renewal unlike the bulk of the DNA-primed RNA polymerase which is rather stable during prolonged inhibition of protein synthesis.

Another possibility is that ribosomal RNA cannot be synthesized in the absence of ribosomal protein or without concomitant ribosomal protein synthesis; otherwise, newly synthesized ribosomal RNA not packaged in ribosomal protein might be rapidly degraded. It is shown above, however, that arrest of protein synthesis with puromycin does not lead to breakdown of pre-existing ribosomal RNA. Kurland and Maaløe[7] have shown that at high concentrations of chloramphenicol there is a progressive decrease with time in the rate of ribosomal RNA synthesis but not of transfer RNA synthesis. They suggest that ribosomal protein is necessary for ribosomal RNA synthesis and that, in the presence of high concentrations of chloramphenicol, ribosomal RNA synthesis proceeds only to an extent allowed by the amount of preexisting ribosomal protein available. Kurland and Maaløe suggest that it was only at chloramphenicol concentrations which do not completely arrest protein synthesis that sufficient "CM particle" protein was produced to allow normal or accelerated rates of ribosomal RNA synthesis.

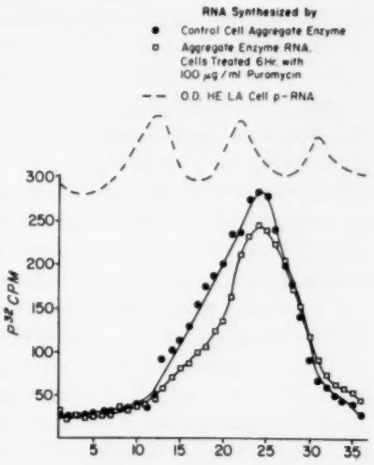


FIG. 4.—Sucrose sedimentation diagram of RNA produced *in vitro* by aggregate enzyme RNA polymerase from normal HeLa cells and from HeLa cells treated with puromycin for 6 hr. Sedimentation of HeLa cell 30S and 18S ribosomal RNA and 4–8S transfer RNA included as a reference. Sedimentation was carried out as in Fig. 1, except that the RNA was heated at 100°C for 30 sec prior to sucrose sedimentation runs (to prevent cosedimentation of nascent RNA annealed to template DNA).

The data presented here in several respects resembles the data of Kurland and Maaløe except that the puromycin effect on mammalian cell RNA synthesis occurs within a fraction of the one day generation time, whereas there is little chloramphenicol depression of *E. coli* RNA synthesis before several generation times have elapsed. Takeda *et al.*[24] have shown that RNA synthesis continues for at least several hr in bacteria treated with puromycin.

The ability of the high uracil-containing RNA to stimulate *in vitro* protein synthesis is being investigated since it shows a number of characteristics expected of informational RNA. If this fraction of rRNA does contain messenger RNA,

the deviation of its base ratios from those of DNA indicate either contamination with other polynucleotides having a high proportion of uracil, or else messenger base compositions in this system differ from those of total DNA.

Penman *et al.*[25] have recently isolated a rapidly labeled RNA that becomes associated with polyribosomes of HeLa cells, and found it to have sedimentation characteristics very similar to the high uracil RNA reported here. The base ratios of their RNA resemble HeLa cell DNA more closely than does the RNA studied here.

Finally, it should be pointed out that the suppression of animal virus RNA synthesis in mammalian cells by inhibitors of protein synthesis does have an analogy in normal cell ribosomal RNA synthesis as shown here. Further work is necessary to determine whether RNA synthesis in each case is suppressed because inhibition of protein synthesis leads to depletion of a labile RNA polymerase or to depletion of a structural protein needed for viral or ribosomal RNA synthesis. The slight decrease regularly observed in HeLa aggregate enzyme RNA polymerase following puromycin treatment shows that at least a portion of the RNA polymerase is sufficiently labile to require renewal within six hr. But it is not yet known whether the RNA polymerase for ribosomal RNA synthesis is the same as, or is different from and more labile than, the bulk of DNA-primed RNA polymerase.

The present study confirms the report of Tamaoki and Mueller[9] that puromycin inhibits ribosomal RNA synthesis. However, they reported continued synthesis of ribosomal precursor RNA after puromycin treatment and they suggested that puromycin acts not by inhibiting polymerization of ribosomal precursor, but by preventing maturation of polymerized ribosomal precursor to 16S and 28S ribosomal RNA molecules. Following the longer periods of puromycin arrest of protein synthesis employed in the present study, it appeared that continuing protein synthesis is required for polymerization of the large ribosomal precursor molecules. Attempts to study these effects in a cell-free system are in progress.

Summary.—Puromycin at a concentration of 100 μg/ml inhibits synthesis of HeLa cell ribosomal RNA, but only slightly inhibits soluble RNA synthesis. Both high molecular weight ribosomal precursor RNA and a smaller rapidly labeled RNA resembling messenger RNA can be separated from ribosomal RNA and transfer RNA by phenol fractionation and detergent treatment of phenol-precipitated nucleoprotein. Puromycin inhibits ribosomal precursor RNA synthesis but does not inhibit synthesis of the smaller rapidly labeled fraction. Thus, phenol fractionation of puromycin-treated cells allowed isolation and sedimentation analysis of this labile RNA which has base ratios very different from that of ribosomal and transfer RNA.

Puromycin inhibition of HeLa cell protein synthesis for 6 hr caused less than 30 per cent inhibition of DNA-primed aggregate enzyme RNA polymerase, indicating that most of the RNA polymerase of HeLa cells is quite stable. However, the decrease that did occur might be enough to account for loss of a separate polymerase concerned with ribosomal RNA synthesis.

RNA produced by DNA-primed aggregate enzyme RNA polymerase from HeLa cells had a broad size distribution with a peak at about 15S. This RNA was similar in size to the rapidly labeled RNA found in the phenol-nonextractable fraction of puromycin-treated HeLa cells.

The author is indebted to Dr. Charles Spotts for stimulating discussions of this problem.

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ON MATERIALS OF HIGH DIELECTRIC CONSTANT

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More than twenty years ago substances of high dielectric constant were constructed in the course of work on certain important radar problems. The substances and their use were kept under secrecy order; a publication of their properties has therefore become possible only recently. Unfortunately, under pressure of war work, only problems of immediate technicological interest could be investigated. The report here given will therefore of necessity be rather sketchy and limit itself to the essential ideas in the construction of the new substances.

Materials of very high dielectric constant at microwave radio frequencies were at that time already known or at least becoming known. We are here referring to compounds of titanium and alkaline earths. These substances, made into ceramics at very high temperatures, consisted mostly of comparatively small plates of irregular

surface and thickness, great hardness, and a dielectric constant exceeding 4,000 which depended quite sensitively on small changes in the temperature and duration of the firing process. Leaving open for the moment the question to which accident this high dielectric constant was due, the other features of the material (small dimensions, irregular thickness, great hardness, unpredictable variation of the dielectric constant with temperature, etc.) ruled it out for any application to radar problems.

Our substances were constructed by forming a microscopic mechanical model of molecules in the ether. The place of the ether was taken by a bland organic binder (mostly rubber-like, as, for example, the GRS of GE). The molecules were represented by metal particles suspended in the binder at so small a concentration that the mixture remained an insulator for D.C. The first attempts with metal grains led to no usefully large dielectric constants. Now these grains of spheroidal form can be looked upon as being a space average of irregularly oriented flakes or filaments. Attempts at calculation did not seem promising, since, for the high dielectric constants aimed at, it could not be hoped that a fair description of the actual case would be given in first approximation, which seemed the only possible way to proceed by analysis. We were therefore led to use not random but sharply oriented flakes or filaments, and to study individually parallel to which direction of the ensuing mixture the dielectric constant became large or small.

To anticipate the final result: by using (for lack of other substances) commercially available flakes, mostly of aluminum but also of iron, copper, silver, etc., which were extremely flat (ratio of thickness to length approximately $^1/_{200}$), and by orienting the flakes parallel to the surface of the mixture which was produced in thin sheets (in a manner to be described later), we obtained without any great difficulty dielectric constants at microwave frequencies as high as approximately 6,300, and with moderate absorption which could easily be regulated by addition of carbon black. We used mixtures up to about 80% by weight of metal. The low absorption indicated that the flakes apparently remained well insulated from each other; no connective chains of metallic contact seemed to exist. With the apparatus at our disposal, we could not measure the dielectric constant normal to the surface, i.e., parallel to the thin dimension of the flake. We were helped out by another division at MIT, which found the dielectric constant in this direction to be of the order 20.

The most striking feature of the new dielectrics is their anisotropy, which can best be understood by study of the method of preparation. The most primitive method consisted of dipping a brush into the binder, and then into a sample of the flaked material. If one painted a piece of paper with this brush, one obtained a dielectric constant of the thin paper plus paint of 1,000. A more refined, and of course much more productive, procedure consisted in filling a de Vilbiss sprayer with an analytically prepared mixture of binder and flakes at suitable concentrations, and then spraying that mixture on any receptor surface. The intensity of spraying could easily be so regulated that one passage of the sprayer over the receptor surface left a thickness of the spray of about one thousandth of a centimeter. The spray solidified and dried during the spraying process. The final material was made up of a superposition of sprayed layers, making the total thickness as great as was desired. The product thus obtained showed, upon close investigation, a triple anisotropy of the dielectric constant parallel and perpendicular to the plane of the sample. The dielectric constant perpendicular to the plane of the sample remained very small

(about 20); the dielectric constant in the plane of the sample parallel to the stroke of spraying was maximal (in one actual case 2,900); the dielectric constant in the plane of the sample perpendicular to the stroke of spraying was reproducibly smaller (in the example quoted about 2,200). If one wanted to obtain the same dielectric constant in both directions of the plane of the sample, one shifted every stroke perpendicular to the direction of the preceding stroke. In this way the sample was built of many strokes of spraying in the x and y directions, and so the dielectric constant in the plane of the sample could be made perfectly homogeneous and isotropic, while the dielectric constant perpendicular to the plane of the sample remained small. We think that there is no other example known in nature where the relative differences between the values of the dielectric constant along the principal axes of a substance are as great as they are here.

The orientation can readily be understood as being due to an effect of surface tension which causes the flakes to be deposited parallel to the receptor surface. We may conclude from this that the dielectric constant reaches a minimum, if the electric vector is parallel to the thin dimension of the flake. There are only a few data available on the variation of dielectric constant (parallel to the surface of the sample) with frequency. If the wavelength was decreased from 10 cm to about 3 or 1.2 cm, the dielectric constant (real part) diminished slightly; the imaginary part of the dielectric constant increased for constant composition. No very complete study was made because the main interest 20 years ago was centered on the production of thin quarter-wavelength samples which, if the wavelength was strongly decreased in this way, became too thin for mechanical reasons if the dielectric constant was left unchanged. We therefore used mixtures with lower metal content and therefore smaller dielectric constant, so as to avoid making the quarter-wavelength sample too thin in the case of small wavelengths. From the point of view of physics, naturally, a closer study of the dependence of dielectric constant on wavelength would be highly desirable.

We want to describe an arrangement with these covers of high dielectric constant (resonant absorbers) which, based on ideas borrowed from the quantum theory of molecules, seems to be of some usefulness. Let us assume that a radio wave becomes diffracted by a static reflecting surface and one or more rotating objects. This is the ideal picture of the scattering from the hull of an airplane together with the scattering from the rotating propellers. Let each rotating object have n parts which carry out equivalent motions (n being the number of blades of one propeller). Then the amplitude of total scattering at every moment is given by the following simple expression:

$$(a_0 + a_1 + \ldots + a_n)^2 \tag{1}$$

This expression contains a periodicity in angle with the period $2\pi/n$ because of the perfect equivalence of the n rotating objects. If the frequency of rotation is given by ω, the frequency of modulation is not ω, but instead equal to $n\omega$. This means in practical language that the frequency of the signal reflected from a plane is modulated not by the propeller frequency but by the product of propeller frequency and number of propeller blades. If now one of the several rotating objects is covered with a thin absorbing layer, then the equivalence is destroyed, and the modulation

will occur with lower frequencies beginning with the fundamental frequency of rotation.

The most interesting features of the materials here described have so far not even been touched upon. The high dielectric constant and the enormous anisotropy could lend themselves to various sorts of experiments not feasible previously. The high dielectric constant would render observable some small effects that occur in the theory of the ponderomotive forces on material dielectrics. Similarly, the great anisotropy should allow the study of optical effects which are present in crystals only to a minute extent due to the small difference between the dielectric constants.

To Professor v. Hippel we want to extend thanks for his help with the measurement of the dielectric constant parallel to the thickness of the samples. Dr. M. H. Johnson, now at Aeroneutronics, and Dr. R. W. Wright, now in charge of the study of absorptive materials for microwaves at NRL, have provided valuable collaboration in the construction and study of the new materials.

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*ON THE NUCLEI OF A SIMPLE JORDAN ALGEBRA**

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The purpose of this note is to provide a reasonably direct proof[1] of the following result.

THEOREM. *The nuclei of any simple Jordan algebra of characteristic not two are all equal to its center.*

We begin our proof with the following trivial remarks. The *right nucleus* of any algebra $\mathfrak{A}$ is the set $\mathfrak{A}_\rho$ of all elements a_ρ of $\mathfrak{A}$ such that $(xy)a_\rho = x(ya_\rho)$ for every x and y of $\mathfrak{A}$. The *left nucleus* $\mathfrak{A}_\lambda$ is the set of all a_λ such that $a_\lambda(xy) = (a_\lambda x)y$. The *middle nucleus* is the set $\mathfrak{A}_\mu$ of all a_μ such that $(xa_\mu)y = x(a_\mu y)$. When $\mathfrak{A}$ is commutative, $\mathfrak{A}_\rho$ and $\mathfrak{A}_\lambda$ are clearly identical. However, $(xy)a = x(ya)$ implies that $x(ay) = (xy)a = (yx)a = y(xa) = (xa)y$. Thus, $\mathfrak{A}_\rho \subseteq \mathfrak{A}_\mu$. Since the *center* $\mathfrak{N}$ of $\mathfrak{A}$ is the intersection of the nuclei in the commutative case, we actually have

$$\mathfrak{N} = \mathfrak{A}_\rho \subseteq \mathfrak{A}_\mu, \tag{1}$$

for all commutative algebras $\mathfrak{A}$.

Let us use the standard notation $xy = xR_y = yx$, where R_y is a linear transformation on $\mathfrak{A}$ called a *right multiplication*. Then it is well known[2] that the identity

$$R_{x(yz)-(xy)z} = (R_zR_z - R_zR_z)R_y - R_y(R_zR_z - R_zR_z) \tag{2}$$

holds. If $y = a$ is in $\mathfrak{A}_\mu = \mathfrak{N}$, the left member of (2) vanishes and (2) becomes

$$(R_zR_y - R_yR_z)R_a = R_a(R_zR_y - R_yR_z) \tag{3}$$

$$(x, y \text{ in } \mathfrak{A}, a \text{ in } \mathfrak{N}).$$

Write

$$b = x(ya) - (xy)a \tag{4}$$

$$(x, y \text{ in } \mathfrak{A}, a \text{ in } \mathfrak{N}),$$

and let $\mathfrak{B}$ be the subspace of $\mathfrak{A}$ of all finite sums of elements of the form $x(ya) - (xy)a$ as in (4). Then

$$b' = [(wy)a]x - [(wy)x]a = x[(wy)a] - [x(wy)]a \tag{5}$$

is in $\mathfrak{B}$, and so are

$$b'' = [(wx)a]y - [(wx)y]a,\ b''' = [(xy)a]w - [(xy)w]a. \tag{6}$$

We now write

$$R_b = R_{x(ya)-(xy)a} = R_y(R_aR_x - R_xR_a) + (R_xR_a - R_aR_x)R_y, \tag{7}$$

and compute

$$wb = b' + [(wx)a - (wa)x]y = b' + q. \tag{8}$$

By (3) we can compute $(R_xR_a - R_aR_x)R_y = R_xR_aR_y - R_a(R_xR_y - R_yR_x) - R_aR_yR_x = R_xR_aR_y - (R_xR_y - R_yR_x)R_a - R_aR_yR_x$. Thus, $q = [(wx)a]y - [(wx)y]a + [(wy)x]a - [(wa)y]x = b'' + [(wy)x]a - [(wa)y]x$. However, $[(wy)x]a - [(wa)y]x = [(yw)x]a - [(ya)w]x$, since $(wa)y = w(ay)$. Thus, $q - b''$ is unaltered by the interchange of w and y, and so $q - b'' = [(yx)a - [(ya)x]\,w - b''' = -wb - b'''$, $q = -wb + b'' - b'''$, and we have proved that

$$2wb = b' + b'' - b'''. \tag{9}$$

This proves that $\mathfrak{B}$ is an ideal of $\mathfrak{A}$. Hence, $\mathfrak{B} = 0$ and $\mathfrak{N} = \mathfrak{R}$ as desired, or $\mathfrak{B} = \mathfrak{A}$.

At this point we do need to introduce some structure theory. We first extend $\mathfrak{R}$ to its algebraic closure Ω and so obtain $\mathfrak{B}_\Omega = \mathfrak{A}_\Omega$. When $\mathfrak{A}$ is *special*, there is a trace function $\delta(x)$ such that $\delta(b) = 0$ for every b in $\mathfrak{B}$.[3] Also, if u is a primitive idempotent of $\mathfrak{A}$, it is true that $\delta(u) = 1$, 2, or $4 \neq 0$, when $\mathfrak{A}$ has characteristic not two. Hence, u cannot be in $\mathfrak{B}_\Omega$, and so $\mathfrak{A} \neq \mathfrak{B}$. When $\mathfrak{A}$ is exceptional and the characteristic of $\mathfrak{F}$ is not 3, we use the trace function $\delta(x)$ equal to the trace of the matrix of R_x. By (2) we see that $\delta(b) = 0$ for every b in $\mathfrak{B}$. But $\delta(e)$ is the dimension of $\mathfrak{A}$ and this is 27. When $\mathfrak{F}$ does have characteristic three, we can compute the middle nucleus of $\mathfrak{A}_\Omega$ directly to obtain our result.

* This work was sponsored in part by the National Science Foundation under NSF grant GP-208.

[1] This result appears in a paper by Robert H. Oehmke and Reuben Sandler entitled "The collineation groups of division ring planes I: Jordan algebras." Their proof was made by using the structure theory for $\mathfrak{F}$ algebraically closed and direct computation.

[2] This basic consequence of the Jordan identity appears as equation (8) in the author's "A structure theory for Jordan algebras," *Ann. Math.*, **48**, 546–567 (1947).

[3] Indeed we have shown that, if $\mathfrak{U}$ is a simple special Jordan algebra over a center $\mathfrak{a}$, and Ω is the algebraic closure of $\mathfrak{a}$, the trace of the elements of $\mathfrak{U}$ *as matrices* satisfies the relation $\delta[x(yz)] = \delta[(xy)z]$. For proof, see the argument on page 410 of the author's 1954 Annals paper entitled "The structure of right alternative algebras."

*ACTIVITIES OF DIRECTLY AND INDIRECTLY ACETYLATED CHYMOTRYPSINS**

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Communicated July 5, 1963

The introduction of one acetyl group into the chymotrypsin molecule by reaction with p-nitrophenyl acetate gives rise to an enzymatically inactive monoacetylated protein that rapidly loses its acetyl group and becomes active again in solutions of dilute alkali or primary alcohols.[1, 2] On the other hand, the well-known polyacetylation of proteins with acetic anhydride, as described by Fraenkel-Conrat, Bean, and Lineweaver,[3] yields active (or potentially active) products with chymotrypsin and chymotrypsinogen in which much of the acetyl remains rather firmly bound. The following paper is meant to give a first account of some observations made on polyacetylated chymotrypsin[3a] and chymotrypsinogen that may be of interest because they indicate that these artifacts behave enzymatically on several counts in very different fashion from their naturally occurring counterparts.

Marked differences between the behavior of derived chymotrypsins and the naturally occurring enzyme were observed by Jansen *et al.*[4] who found the pH optimum of esterolysis[4a] to be altered by acetylation. They also observed that esterolytic activity is much less reduced by oxidation with periodate than is proteolytic activity. The chemical modifications made by Vallee *et al.*[5] on carboxypeptidase A have led to even more striking changes in the properties of that enzyme. The peptolytic activity may be caused to disappear altogether, while at the same time the esterolytic activity increases manyfold.

Multiple acetylation of chymotrypsin led in our hands to a product of mediocre activity, compared to the original enzyme, on both TEE and protein. However, when a solution was maintained at neutrality for some time, its esterase activity approximately doubled and thus became about equal to that of the natural enzyme. The proteolytic activity remained unchanged.

Observations on chemically modified chymotrypsinogens, including acetylated chymotrypsinogen, have been reported by Wilcox in a series of papers.[6–9] They leave no doubt that in many instances such modified proteins yield active ester-splitting enzymes on treatment with trypsin. In the case of the acetylated zymogen, the observed esterolytic activity was as high as that obtained from natural chymotrypsinogen under like conditions.[7]

Since some of the groups in active chymotrypsin, including the so-called "active center" itself, are evidently not available for reaction in chymotrypsinogen, we thought it might be of interest to compare the active protein obtained by the direct acetylation of chymotrypsin with that obtained by activation of the previously acetylated zymogen. It was found that acetylated chymotrypsinogen differed from the naturally occurring protein in its behavior with trypsin. Unlike the natural protein, it formed little or no active enzyme with trypsin at pH 7.0-7.2, unless an electrolyte was also present in appreciable concentration or, alternatively, some natural (i.e., not acetylated) chymotrypsin. Moreover, the product of activation differed from both natural and directly acetylated chymotrypsins in that its estero-

lytic activity (measured with TEE) was about twice that observed with the natural protein, although proteolytic activity remained at the same low level as that of the directly acetylated enzyme.

Materials and Methods.—Chymotrypsin was prepared from 8 times recrystallized chymotrypsinogen as described by Northrop, Kunitz, and Herriott.[10] Both proteins were acetylated at 0° in half-saturated sodium acetate solution with 1.3 times their weight of acetic anhydride added over one hr. Thereafter, the material was dialyzed and finally dried *in vacuo* while frozen. Titration of the resulting products with NaOH in the presence of formaldehyde (formol titrations) showed that acetylation had caused a loss of about 80% of the titratable groups of the chymotrypsinogen and 71% of those in the chymotrypsin used. The molecular weights of these derivatives, as well as of the original proteins, have all been taken at the approximate value of 25,000.

L-tyrosine ethyl ester (TEE) was recrystallized from commercial material and checked with respect to optical activity. Other reagents were good grade commercial products.

The esterolytic activity of chymotrypsin was determined by the initial rate of hydrolysis of 20 μmoles of L-tyrosine ethyl ester in 2.0 ml of 0.025 *M* $CaCl_2$, pH 6.25 at 25° as measured by essentially continuous titration with 0.10 *N* NaOH from a microburette. The same apparatus was used for the titration of the acetylated proteins in μ-molar quantities. The milk-clotting capacity[11] of the enzyme was determined at 40° on 10 ml portions of a 10% solution of dried skim milk, pH 6.2–6.3, made 0.01 *M* with $CaCl_2$ shortly before use. Under these conditions, both milk-clotting and esterolytic activities are for some time linear functions of the quantity of enzyme employed. Specific activity (Sp. Act.) is defined as moles of ester split per minute per gm of protein. Milk-clotting activity, likewise a constant for a given preparation, is expressed as $K = 1/(t)\,(mg)$, where t is clotting-time in minutes and mg, the milligrams of enzyme present. The digestion of casein is reported as the increase in optical density at 280 mμ obtained with 100 μg of enzyme protein by the procedure of Kunitz,[12] except that the reaction time was 15 min at 25°.

The action of hydroxylamine was measured by the method of Hestrin[13] as slightly modified for use with proteins.[14]

Activation of Acetylated Chymotrypsinogen.—Trypsin was added to the slightly turbid solution of the acetylated zymogen in water, followed by dilute NaOH until the pH remained constant at 7.0.[14a] Aliquots of such a solution were then mixed with solutions of the additions to be tested, also at pH 7.0, in appropriate concentrations. Experiments carried out at 5° contained one part of trypsin protein per thousand of zymogen; those at room temperature (25°) were made with one part of trypsin to 200 of zymogen. The former series required several days to reach full activity in some cases; the latter, only a few hours. No differences in principle were seen between the two series of experiments. It was observed that all salts were not equally efficient promoters of activation. Maleate and citrate buffers, 0.05 *M*, were particularly satisfactory, whereas 0.3 *M* NaCl and 0.5 *M* Na acetate did not produce as rapid activation. Table 1 gives some typical examples. The substitution of natural chymotrypsin for any electrolyte is noteworthy, as is the failure of acetylated chymotrypsin to act in a similar fashion.

The esterolytic activity developed from the acetylated zymogen usually amounted to 150–200 per cent of the value found for the natural enzyme or for the directly acetylated enzyme after neutralization. It was not accompanied by an increase in milk-clotting (or casein-digesting) power. The rate at which these two functions of the enzyme developed during the activation process is of interest because the natural and the acetylated chymotrypsinogens differed in this respect, as Table 2 shows.

An inspection reveals that this difference in behavior between natural and acetylated zymogens is not owing to the faster development of milk-clotting power in the

TABLE 1

TRYPTIC ACTIVATION OF ACETYLATED CHYMOTRYPSINOGEN

Addition	Temp.	Specific Activity (TEE)* Hr 0.5	1.0	1.5	20	40	66
Water only	5				0	0	0
$CaCl_2$, 0.025 *M*	5				0.014	0.071	0.069
Maleate, 0.10 *M*	5				0.050	0.071	0.079
NaCl, 0.30 *M*	5				0.033	0.063	
Na acetate, 0.50 *M*	5				0.041	0.062	
Water only	25	0.006	0.008	0.009			
$(NH_4)_2SO_4$, 0.05 *M*	25	0.034	0.055	0.057			
Maleate, 0.05 *M*	25	0.038	0.057	0.069			
Natural chymotrypsin† (Sp. Act. 0.040)	25	0.058	0.058	0.058			
Directly acetylated chymotrypsin†	25			0.003			
The activation product of acetylated chymotrypsinogen†,‡	25			0.007			

* Based on weight of the zymogen used for activation.
† Ten % of weight of zymogen. Correction has been made for the additional activity thus introduced.
‡ Made by rapid activation in maleate buffer as shown above, then dialyzed.

acetylated case, thus keeping pace with the greater esterolytic activity formed, for the reverse is true; the esterolytic activity developed more slowly, although eventually it went further. Only at the very beginning was there any resemblance between the two systems.

TABLE 2

RATE OF TRYPTIC ACTIVATION OF NATURAL AND OF ACETYLATED CHYMOTRYPSINOGEN

Time, min	Activity Developed* Chymotrypsinogen Milk K	TEE Sp. Act.	Ratio TEE Milk × 10^2	Acetylated Chymotrypsinogen Milk K	TEE Sp. Act.	Ratio TEE Milk × 10^2
5	1.4	0.035	2.5	0.6	0.018	3.0
10	2.4	0.049	2.0	1.0	0.030	3.0
30	2.8	0.044	1.6	2.2	0.062	2.8
60	2.6	0.040	1.5	2.4	0.070	2.9
90	2.4	0.040	1.7	2.4	0.070	2.9

* Based on weight of zymogen.

There is no need to invoke the existence of two different sites in the protein in explanation of these results. The behavior of the natural zymogen is consistent with the formation of a series of intermediates such as what is known to form under these conditions.[15] By the same token the acetylated zymogen seems not to undergo the same type of progressive degradation. This could account for its high (and stable) esterolytic activity, but not for the observed low proteolytic power. However, it is evident from the observation of Antony[16] that proteolytic activity in heavily acetylated chymotrypsin may be influenced by groups stable at pH 8, but labile with hydroxylamine (obviously other than the group(s) in question here).

Directly Acetylated Chymotrypsin.—As prepared by us, the protein formed an opalescent solution in water, pH 4.5–4.7. About 4–5 equivalents of NaOH per mole of protein were required to bring the solution to neutrality. Thereafter, a slow liberation of acid took place, lasting about an hour and amounting to one molar equivalent. The specific esterolytic activity was approximately doubled by this treatment, thus reaching about the same level as that of the natural enzyme, but

still only half or two-thirds the activity shown by the product of activation of acetylated chymotrypsinogen. The same result was obtained by storing a solution of the protein in neutral maleate buffer overnight at 5°. Table 3 gives comparable values for esterolysis, milk-clotting, and also casein digestion. The milk-clotting power of the directly acetylated enzyme did not increase on neutralization. The increase in ester-splitting activity is permanent. It is not reversed by reacidification.

TABLE 3

ESTEROLYTIC AND PROTEOLYTIC ACTIVITIES OF ACETYLATED PROTEINS

	TEE Sp. Act.*	Milk K*	Casein digestion*
α-Chymotrypsin	0.040	3.1	1.11
Directly acetylated chymotrypsin, pH 4.6	0.018	1.7	—
Directly acetylated chymotrypsin, 20 hr in .05 *M* maleate, pH 7.0	0.040	1.8	0.78
Activation product of acetylated zymogen in .05 *M* maleate, pH 7.0	0.064	2.6	0.78

* See text under *Methods*.

Action of p-Nitrophenylacetate and Hydroxylamine on Acetylated Chymotrypsins.— The behavior of directly acetylated chymotrypsin strongly suggests that the active site of the enzyme has remained partly acetylated throughout the later steps (dialysis and lyophylization) in the preparation of the protein. Ordinary monoacetyl chymotrypsin is unstable under such conditions, but it may be postulated that the presence of other acetyl groups in the protein slows down the decomposition of this particular group through something akin to steric hindrance. To test this hypothesis, the protein was treated with p-nitrophenylacetate under conditions previously described.[14] The burst of free nitrophenol that takes place when the protein and this reagent are mixed may be taken as a measure of the "active" group that is available for acetylation. A sample (25 mg) of directly acetylated chymotrypsin was dissolved in 0.10 *M* PO_4 buffer, pH 6.2, and tested in this way when the solution was 10 min old, and also after 20 hr at 5°. Table 4 (Items 5, 6, and 7) shows that a marked increase in the burst of nitrophenol accompanied the increase in esterolytic activity.

The presence of an active acetyl group was also indicated by the reaction with

TABLE 4

ACTION OF P-NITROPHENYLACETATE AND HYDROXYLAMINE ON ACETYLATED CHYMOTRYPSINS

	Hydroxamic acid* μmoles/25 mg		Burst of p-NO_2 phenol	
	pH 6.0	pH 11.2		Sp. Act. TEE
(1) Acetylated chymotrypsinogen	0.8	2.9	—	—
(2) Same, after activation‡	0.3	1.3	0.90	0.078†
(3) Same, activated‡ after treatment with 2 *M* hydroxylamine at pH 6				0.073†
(4) Same,‡ except hydroxylamine at pH 11.2				0.065†
(5) Directly acetylated chymotrypsin (pH of solution, 4.6)	1.2	2.2	0.52	0.017
(6) Same, after 20 hr, 5°, at pH 7.0	0.1	1.1	0.82	0.030
(7) α-Chymotrypsin	0.2	0.2	0.80	0.040
(8) α-Chymotrypsinogen	0.2	0.2	—	—

* After subtraction of the "blanks" (0.2) shown below for the unacetylated proteins (items 7 and 8).
† Based on weight of zymogen used.
‡ In 0.05 *M* maleate, pH 7.0, at 25° for 2 hr.

hydroxylamine. Table 4 shows the amount of hydroxamic acid formed at pH 6.0 and 11.2 by several typical acetylated preparations.

The presence in the acetylated zymogen of so much active acetyl is surprising, but it has been observed in three separate preparations. However, this appears to have no connection with the increase of esterolytic activity reported here; for although the *removal* (at pH 7) of one O-acetyl (or imidazole-acetyl) practically doubled the esterolytic activity of the directly acetylated protein, treatment of the acetylated zymogen with hydroxylamine at either pH 6 or pH 11.2 had no effect on the esterolytic activity of the activation product. Therefore, the acetyl group responsible for this increased activity is not apt to be attached to hydroxyl in tyrosine or to imidazole[17] but is in all probability an N-acetyl configuration. The effect of such a substituent might well be a negative one. Instead of increasing the activity, it could simply prevent a loss thereof. One is inevitably reminded of the breakdown of the π-chymotrypsin of Jacobsen[18] to proteins of lower proteolytic activity, and wonders whether an appropriate substituent group in the zymogen might not hinder that change.

The Role of Chymotrypsin in the Activation of Acetylated Chymotrypsinogen.—The process of activation, which always involved trypsin (soy-bean trypsin inhibitor prevented it), depended also on the action of electrolyte such as sodium chloride or, alternatively, on the action of natural chymotrypsin. So far as present observations have gone, the end result appears to be the same, but this can hardly be the case. Both acetylated zymogen and directly acetylated chymotrypsin rapidly liberated acid groups titratable at pH 7.0 in 0.5 *M* NaCl. But we found that other proteins, for example, bovine serum albumin, acted the same way. Such changes may bespeak an alteration in the tertiary structure of the protein.[19] Chymotrypsin, however, liberates no acid rapidly from either acetylated protein. Its action could be expected rather on the primary structure. This viewpoint is borne out by the observation that chymotrypsin attacked the acetylated zymogen and rendered it activable by trypsin in the absence of electrolyte. This was shown by treatment of acetylated chymotrypsinogen with chymotrypsin at pH 7.0 in the absence of trypsin and salts. A tenfold excess of diisopropyl fluorophosphate (over the chymotrypsin) was then added. A test 30 min later with tyrosine ester showed that all chymotryptic activity was gone. Thereafter, the addition of trypsin alone to the isolated zymogen produced a specific activity of 0.067 in 2 hr at 25°.

Summary.—The introduction of a number of acetyl groups into α-chymotrypsin by treatment with acetic anhydride reduced both proteolytic and esterolytic activities. The latter activity, but not the former, was restored, usually to about the level found for the unacetylated enzyme, by standing in neutral or slightly alkaline solution. The protein lost one active acetyl group during this treatment, thus acting like monoacetyl chymotrypsin prepared with nitrophenylacetate.

Acetylated chymotrypsinogen could be activated by trypsin, but only under special conditions that are not necessary for the naturally occurring protein. The presence of an electrolyte or else the addition of some natural (i.e., unacetylated) chymotrypsin was required. When either of these conditions was fulfilled, the end product of activation showed about the same proteolytic activity as directly acetylated chymotrypsin, but the esterolytic activity was nearly double that found for either the directly acetylated or the natural enzyme. The effect of natural

chymotrypsin in this activation was at least in part on the acetylated chymotrypsinogen itself before the attack thereon by trypsin. The failure of hydroxylamine to change the potential activity of the zymogen indicated that the markedly increased esterase activity of the activation product is owing to the presence of an N-acetyl derivative. A simple viewpoint that fits the presently known facts is that such an N-acetyl group is so situated as to offer "steric hindrance" to the rearrangement or further degradation of the enzyme to a less active form.

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† A laboratory of the Western Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture.

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[11] We have in general regarded the outstanding milk-clotting activity of chymotrypsin as a measure of its proteolytic activity. The digestion of casein[12] has also been used here in some instances as an additional criterion, but it should be remembered that all such methods largely depict peptolytic rather than proteolytic activity. The measurement of actual protein-splitting, particularly in the early stages, has not as yet been achieved with satisfactory accuracy except (perhaps) by the measurement of viscosity. Gelatin is already too greatly modified to serve as a typical protein. Casein with added calcium salts is, however, an excellent substrate of chymotrypsin.

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*DEPRESSED INCORPORATION OF THYMIDINE-H³ INTO DEOXYRIBONUCLEIC ACID FOLLOWING ADMINISTRATION OF 7,12-DIMETHYLBENZ(a)ANTHRACENE**

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In the experiments to be described, it was observed that the administration of 7,12-dimethylbenz(a)anthracene (7,12-DMBA) prior to the injection of tritium-labeled thymidine (TdRH³) considerably depresses the incorporation of tritium into rat testis, intestine, and adrenal. Recovery from the effect of 7,12-DMBA occurs in intestine within 48 hr but in testis the inhibitory action of a single dose of 7,12-DMBA persists longer. Fractionation of the testis demonstrated that the decrease in tritium content is associated entirely with the perchloric acid-insoluble fraction.

7,12-DMBA is of special interest for several reasons. (1) A single dose evokes mammary cancer selectively, invariably, and rapidly in the rat under conditions which are easily satisfied.[1] (2) A single dose of the hydrocarbon invariably induces massive and selective necrosis of two of the zones of adrenal cortex[2] of adult, but not of infant, rats.[3] (3) In male rats, a solitary injection of 7,12-DMBA inflicts selective damage in the testicular cells which actively synthesize deoxyribonucleic acid (DNA) and only in cells of this class.[4] Death of male germinal cells is not sudden, but occurs only after the injured cells have undergone one or several mitotic divisions. These spectacular biological effects of 7,12-DMBA made it desirable to investigate the influence of this compound upon the incorporation of thymidine into the DNA of testis and other tissues.

Experimental.—Male rats of Sprague-Dawley strain were used exclusively; there were 4 animals in each group. Each experiment was repeated on 3 separate occasions with similar results. Tritium-labeled thymidine,[5] specific activity of 3 c/mmol, was diluted with aqueous sodium chloride (0.9%), and a 1 ml dose, corresponding to approximately 1 μc/gm of body weight, was injected into a caudal vein. 7,12-DMBA, mp 122–3°, purified by Florisil chromatography and recrystallized from acetone-ethanol, was made up in lipide emulsion[5] and injected once only into a caudal vein at various time intervals before or after the TdRH³. Infant rats, age 25 days weighing 52–70 gm, received 2 mg of 7,12-DMBA in 0.4 ml of emulsion; adult rats, age 50 days weighing 200–220 gm, received 5 mg of 7,12-DMBA in 1 ml of emulsion.

The animals were killed by decapitation, and testis, adrenal, and a segment of terminal ileum were excised and weighed. For determination of the total volatile and nonvolatile radioactivity, each tissue was dried from the frozen state; the evaporated water was collected in a cold trap and counted directly, whereas the dry residue (triplicate samples for testis) was analyzed for tritium by the combustion procedure described previously.[6] For determination of tritium in the acid-soluble and acid-insoluble fractions, the weighed testis was homogenized for 3 min in 4 ml of ice-cold 0.5 *N* perchloric acid containing 0.5 mg of nonradioactive thymidine per ml. All procedures after weighing were were carried out at a temperature of 4° or

lower. The homogenate was centrifuged at 10,000 *g* for 15 min at 0°, the supernatant decanted, and the precipitate washed twice by trituration with the cold perchloric acid-thymidine solution. The supernatants and washings from each sample were combined and diluted to 25 ml with distilled water; aliquots were taken for drying and combustion. The precipitate was dried first in air and then in vacuum, and the residue was analyzed for tritium in triplicate by the combustion technique. The results are expressed in μc per gm of original tissue.

Tissues were fixed in Bouin's fluid for histological examination, and paraffin sections were prepared. For radioautography the dip-coating method of Joftes[7] was employed. The slides were exposed for 6 weeks before development.[5]

Results.—The ability of 7,12-DMBA to depress the incorporation of tritiated thymidine into the testis and adrenal of mature rats is illustrated by the results in Table 1. In control animals the uptake of radioactivity in adrenal is about twice

TABLE 1

DEPRESSED INCORPORATION OF TRITIATED THYMIDINE INTO TESTIS AND ADRENAL CAUSED BY 7,12-DMBA

Injection of 7,12-DMBA	Nonvolatile radioactivity		Volatile radioactivity	
	Testis	Adrenal	Testis	Blood
Controls	0.15	0.33	0.62	0.61
4 hr after TdRH³	0.13	0.25	0.68	0.68
4 hr before TdRH³	0.05	0.14	0.84	0.85

Male rats, age 50 days weighing *ca.* 220 gm, were injected with 5 mg of 7,12-DMBA before or after injection of 200 μc of TdRH³. Control animals received TdRH³ only. Animals were sacrificed 24 hr after TdRH³. Results are expressed in μc per gm wet weight.

that in testis on a wet weight basis, but because of the low solid content of testicular tissue, the concentration of radioactivity on the dry weight basis actually is slightly higher in testis than in adrenal. In both tissues the level of radioactivity present 24 hr after thymidine administration is depressed only slightly when 7,12-DMBA is injected after TdRH³; but when the hydrocarbon is administered 4 hr before thymidine, the incorporation of radioactivity is decreased to about $^1/_3$ the control values.

A similar effect of 7,12-DMBA on the incorporation of tritiated thymidine by testis and by intestine is seen in the young rat (Table 2). In these animals the

TABLE 2

DEPRESSED INCORPORATION OF TRITIATED THYMIDINE INTO INTESTINE AND ACID-INSOLUBLE FRACTION OF TESTIS CAUSED BY 7,12-DMBA

Injection of 7,12-DMBA	Testis			Ileum
	Total	Acid-insoluble	Acid-soluble	Total
Controls	0.39	0.34	0.01	2.21
4 hr after TdRH³	0.35	0.30	0.02	1.97
4 hr before TdRH³	0.11	0.09	0.02	0.20

Male rats, age 24 days weighing *ca.* 60 gm, were injected with 2 mg of 7,12-DMBA after or before receiving 60 μc of TdRH³. Control animals received TdRH³ only. Animals were sacrificed 21 hr after TdRH³. Values represent nonvolatile radioactivity expressed in μc per gm wet weight of original tissue. Total radioactivity was determined on left testis; acid-soluble and acid-insoluble radioactivity on right testis of the same animals.

uptake of tritiated thymidine by the growing testis is considerably greater than that seen in the older animals; even so, the concentration of radioactivity in intestine is five times that in testis. Again, the administration of 7,12-DMBA after TdRH³ slightly depressed the incorporation of radioactivity into both testis and intestine,

while injection of the hydrocarbon 4 hr before tritiated thymidine caused a striking depression to less than $^1/_3$ the control level in testis and to $^1/_{10}$ the control level in intestine.

In the case of testis, the effect of 7,12-DMBA was shown to be exclusively on the incorporation of radioactive thymidine into the acid-insoluble components of the tissue. As seen in Table 2, as well as in Table 4, the acid-soluble radioactivity comprises only a small fraction of that present in testis, and its level is essentially unaffected by administration of the hydrocarbon. The acid-insoluble radioactivity is markedly depressed by the action of 7,12-DMBA, lowering the total amount of nonvolatile radioactivity present.

Radioautography: Evidence that the incorporation of thymidine into DNA is being inhibited by the action of 7,12-DMBA is provided by radioautographic examination of testis from rats receiving tritiated thymidine. At 1, 4, and 21 hr after the injection of TdRH^3, radioautographs showed that tritium was present in spermatogonia of all types and in resting spermatocytes but in no other cells in the seminiferous tubule. This radioactivity was heavily concentrated in the nuclei. In rats which received 7,12-DMBA 4 hr before TdRH^3, fewer testis tubules were labeled, and there were fewer grains above the nuclei of the labeled cells (Table 3). When 7,12-DMBA was injected 4 hr after TdRH^3, radioautography revealed that the decrease of tritium incorporation was slight.

TABLE 3

DISTRIBUTION OF RADIOACTIVITY IN TESTIS OF RATS INJECTED WITH 7,12-DMBA AND TdRH^3

Injection of 7,12-DMBA	Testis tubules			Spermatogonia
	No. counted	No. labeled	%	Grains per cell
Controls	527	219	42	22 ± 10
4 hr after TdRH^3	496	193	39	21 ± 10
4 hr before TdRH^3	423	96	23	11 ± 4

±, standard deviation.

Male rats, age 24 days weighing *ca.* 60 gm, were injected with 2 mg of 7,12-DMBA, after or before injection of 60 μc of TdRH^3. Control animals received TdRH^3 only. Animals were sacrificed 21 hr after TdRH^3. The distribution of radioactivity was determined in radioautographs exposed for 6 weeks.

Effect on volatile radioactivity: In rats receiving tritiated thymidine, the blood and tissues contain a considerable amount of volatile radioactivity which may greatly exceed the nonvolatile radioactivity incorporated (Tables 1 and 4). This

TABLE 4

DURATION OF 7,12-DMBA EFFECT ON THYMIDINE UPTAKE BY TESTIS AND BY INTESTINE

TdRH^3 on day	Time DMBA before TdRH^3	Testis			Ileum	
		Nonvolatile				
		Acid-insoluble	Acid-soluble	Volatile	Nonvolatile	Volatile
25	Controls	0.27	0.02	0.52	2.17	0.35
25	1 day	0.08	0.02	0.69	0.62	0.62
26	2 day	0.08	0.02	0.61	2.59	0.60
29	5 day	0.10	0.02	0.54	1.80	0.48
31	7 day	0.07	0.02	0.52	1.81	0.48

Male rats, age 24 days weighing *ca.* 60 gm, were injected with 2 mg of 7,12-DMBA and at various times thereafter with TdRH^3 (1μc/gm). Control animals received TdRH^3 only. Animals were sacrificed 4 hr after TdRH^3. Results are expressed in μc per gm wet weight of original tissue.

volatile tritium probably represents tritiated water formed from the metabolic degradation of the nucleoside. Treatment of the animal with 7,12-DMBA causes a significant increase in the concentration of volatile radioactivity in all tissues examined. It would appear that when the rapid incorporation of tritiated thymi-

dine into cells is inhibited by 7,12-DMBA, more of the nucleoside is available for metabolic breakdown, leading to an increased concentration of tritiated water in the body fluids.

Time-dependence of 7,12-DMBA action: In the foregoing experiments it was found that 7,12-DMBA injected 4 hr before TdRH[3] causes a profound inhibition of the incorporation of radioactivity into tissues during a 21–24 hr period thereafter, whereas 7,12-DMBA injected 4 hr after TdRH[3] shows only a slight effect. Since extensive thymidine incorporation into the acid-insoluble components of testicular tissue appears to take place quite rapidly, the importance of allowing the hydrocarbon to reach the tissue before the thymidine was studied further. Young rats were injected with 7,12-DMBA at various intervals from 5 min to 24 hr prior to TdRH[3] administration, and the animals were sacrificed just one hr thereafter. It was found (Fig. 1) that 7,12-DMBA injected up to one hr before thymidine has essentially no effect on the incorporation in testis of radioactivity taking

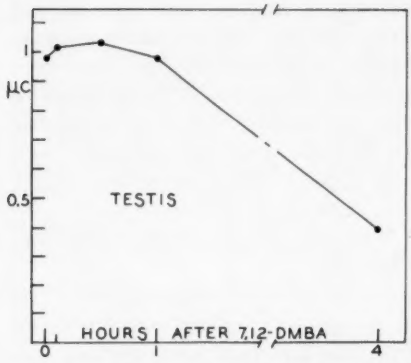

FIG. 1.—Time required for 7,12-DMBA to depress thymidine incorporation into the acid-insoluble fraction of testis. Rats age 26 days were injected with 58 μc of TdRH[3] at various intervals after receiving 2 mg of 7, 12-DMBA; the 0 hr group received no hydrocarbon. Tissues were harvested at 1 hr after injection of TdRH[3]. Ordinate is μc per gm dry acid-insoluble residue.

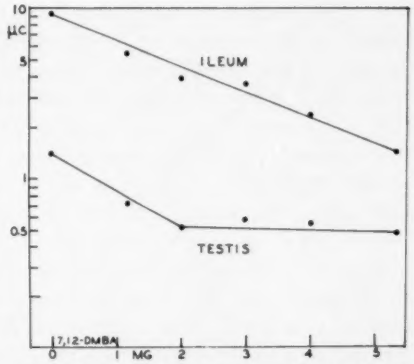

FIG. 2.—Effects of dosage of 7,12-DMBA upon incorporation of tritiated thymidine into nonvolatile fractions of ileum and testis. Rats, age 32 days and weighing *ca.* 100 gm, were injected with various amounts of 7, 12-DMBA 20 hr before receiving 101 μc of TdRH[3]. Tissues were harvested 4 hr after TdRH[3]. Ordinate is μc per gm of dry tissue.

place in the first hr, whereas hydrocarbon administered 4 hr, as well as 24 hr, prior to TdRH[3] markedly depresses tritium incorporation. After intravenous injection it would appear either that 7,12-DMBA takes a longer time to reach the tissue than does thymidine or, more likely, that the hydrocarbon must interact with cells for a certain period of time before its full effect in depressing thymidine incorporation is realized.

The duration of the effect of 7,12-DMBA on thymidine incorporation varies considerably in different tissues. As seen in Table 4, a single dose of 7,12-DMBA depresses for at least 7 days the ability of testis to incorporate thymidine, whereas recovery from the effect of the hydrocarbon in intestine appears to take place within 48 hr. The effect of 7,12-DMBA in increasing the volatile radioactivity in the

tissue fluids persists for at least 2 days, after which there is a return toward the control levels.

Effect of 7,12-DMBA dosage: The dosage of 7,12-DMBA required to depress incorporation of tritiated thymidine was investigated in rats age 32 days which are intermediate in size between the two types of animals used in the previous experiments. A difference was found between the sensitivity of ileum and that of testis. In ileum, increasing the dose of 7,12-DMBA 1–5 mg resulted in a progressive decrease in the concentration of nonvolatile radioactivity (Fig. 2); but in testis, maximum depression was reached with a 2 mg dose of 7,12-DMBA, and larger amounts of hydrocarbon did not show any greater effect. On the basis of these results, it would appear that the doses of 7,12-DMBA employed in the previous experiments were sufficient to elicit maximal depression of thymidine into testis.

Discussion.—From the foregoing experiments it is clear that 7,12-DMBA has a striking ability to depress but not to eliminate completely the rapid incorporation of radioactivity of tritiated thymidine into at least three tissues. In testis the bulk of the radioactivity appears in the washed perchloric acid-insoluble fraction, and it is in this fraction where effect of 7,12-DMBA is evident. Since it has been established that thymidine is not incorporated directly into ribonucleic acid[8, 9] and since the entry of the tritiated methyl group of thymidine into protein or lipide should be negligible, it is probable that this radioactivity is associated with deoxyribonucleic acid. Moreover, in radioautographs of testis the bulk of the radioactivity is seen in the germinal epithelium and is concentrated in the nuclei of the cells[10, 11] which actually synthesize DNA; it is not present in cells which proliferate by meiosis. Therefore, it is reasonable to assume that it is the incorporation of thymidine into DNA which is inhibited by the action of 7,12-DMBA.

At present, the mechanism of the inhibitory action of 7,12-DMBA is obscure. It would appear that the hydrocarbon needs to interact with cellular constituents for a period of a few hr before depression of thymidine incorporation is realized, and that different tissues recover from the effect of 7,12-DMBA at considerably different rates. Further, it appears that by preventing the incorporation of tritiated thymidine into cells 7,12-DMBA causes more of the administered nucleoside to undergo metabolic degradation with liberation of tritium from the methyl group.

Whether the effect of 7,12-DMBA on thymidine incorporation bears any direct relation to its carcinogenic action is uncertain, since the tissues studied are not those in which cancers are induced. However, it is possible that the multiplicity of biological effects exhibited by 7,12-DMBA represent different manifestations of a common fundamental action on nucleic acid formation and integrity: this consideration affords a stimulus for further experimentation.

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*METABOLICALLY LABILE DEOXYRIBONUCLEIC ACID**

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DNA prepared from a variety of growing plant tissues may be resolved on a methylated albumin column into two distinct fractions. A full account of the preparative procedure and physical characterization of the components will be published elsewhere. For the purpose of this communication, the following points are to be noted: tissues are disintegrated in cold 95% ethanol and extracted with 5% sodium lauryl sulfate (w/v) in 0.05 *M* sodium citrate at a pH of 7.4. The nucleic acids are precipitated with two volumes of ethanol, re-extracted with 2.5 *M* sodium chloride, deproteinized with chloroform:amyl alcohol, and digested with ribonuclease.[1] The product is dissolved in 0.1 *M* NaCl:0.05 *M* potassium phosphate (pH, 6.8), and loaded on a methylated albumin column.[2, 3] By stepwise elution with increasing concentrations of NaCl, two DNA fractions are obtained, one eluting at 0.4–0.5 *M* NaCl, the other at approximately 0.65 *M*. Viscometric analyses indicate their respective molecular weights to be of the order of $2–3 \times 10^5$ and $4–6 \times 10^6$. Both fractions show melting profiles typical for a double-stranded helix, but the maximum increase in optical density of the low molecular weight form is about 31–33%.

The proportion of the two forms varies with type of tissue and with physiological state. Male germinal tissue contains negligible amounts of the low molecular weight form; ovules have not been analyzed. Growing regions of root and leaf, including zones of differentiation, may have as much as 20% of their total DNA in the low molecular weight form. Dormant embryos in wheat seeds have about 10%, but the value rises sharply on induction of germination. Accurate estimates have not yet been made for mature tissues because of possible complications arising from endogenous breakdown of high molecular weight DNA. It will become apparent from the experiments here reported that low molecular weight DNA isolated from growing tissues is not a breakdown product.

Experimental Results.—Wheat seeds (var. Selkirk) were germinated and grown in Hoagland's solution with the phosphate component at half strength. When the leaves reached 2–3 inches in height, P^{32}-inorganic phosphate was added to the medium. Roots were removed from plants after various periods of exposure to the isotope, and also from plants which, following exposure, were thoroughly washed

with 0.1 *M* phosphate and returned to Hoagland's medium. Leaves were not used in these experiments because of their sluggish response to isotope addition or removal. Root DNA was prepared as described. The fractions removed from the column were concentrated by lyophilization, treated overnight with 0.2 *N* NaOH at 37°C, and again at 70°C for 20 min to remove possible traces of RNA. Neither low nor high molecular weight DNA were degraded under these conditions. Recoveries were virtually quantitative for the high molecular weight form and nearly so for the low molecular weight. In the course of experimentation it was found that losses (10–20%) could be avoided by maintaining the concentration of low molecular weight DNA at relatively high levels (0.25 mg/ml). The DNA content of alkali-treated fractions was determined by ultraviolet absorption and by the diphenylamine reaction.

Typical results of such experiments are given in Figure 1. The principal feature of these results is the contrasting behaviors of the two DNA forms with respect to P^{32} turnover. High molecular weight DNA behaves in the expected fashion; the specific activity of P^{32}-DNA decreases only slightly following exposure of roots to P^{31}-phosphate. By contrast, the specific activity of low molecular weight DNA decreases to the vanishing point following the same exposure to P^{31}. Precise studies of RNA turnover during these treatments were not made, but if ribonuclease treatment prior to column chromatography was omitted, the RNA released by alkaline hydrolysis from the low molecular weight DNA fraction showed no comparable decrease in radioactivity following exposure to P^{31}.

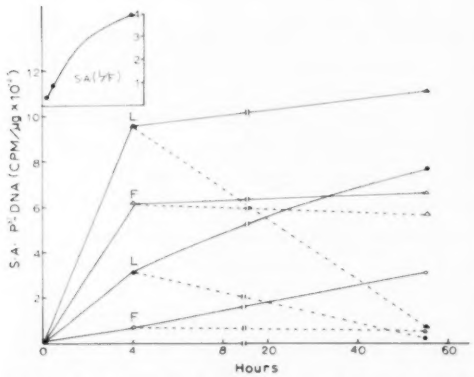

FIG. 1.—Changes in specific activity (S.A.) of fibrous (F) and low molecular weight (L) DNA in growing wheat roots following exposure to P^{32}-phosphate. The terms "fibrous" and "high molecular weight" will be used interchangeably. Dashed lines represent values after "chasing" with P^{31}-phosphate. The inset is discussed in the text; the abscissae are the same as those for the main graph.

The inset in Figure 1 shows the relative specific activities of the two types of DNA following brief exposures of wheat roots to isotope. This particular pattern, however, is not consistently obtained. Physiological conditions influence the pattern, and so too, undoubtedly, the nature of precursor pools. Results such as these are nevertheless difficult to explain in terms of one form of DNA being the precursor of the other. Tentatively at least, one may assume that the two forms of DNA are functionally distinct. Further experiments were undertaken to test this assumption and also to establish more soundly that the material being studied in the low molecular weight fraction was, in fact, DNA.

The two types of P^{32}-DNA were prepared from corn roots, digested with crystalline deoxyribonuclease followed by purified snake venom phosphodiesterase,[4] calf thymus DNA being added to each of the fractions as a carrier. The deoxy-

nucleotides were fractionated on a Dowex-formate column.[5] The plot of radioactivity tracked the plot of ultraviolet absorption as shown in Figure 2. Most of the hydrolytic products from the two plant preparations were the same as those from calf thymus DNA. These results thus further establish that the labeled material being studied is DNA. A second and important characteristic is, however, revealed by this experiment. The total radioactivities in each of the deoxynucleotide fractions is listed in Table 1. It may be seen that, although the composition of high molecular weight DNA falls in the class typical for higher plants, the composition of the low molecular weight form is very different. Assuming a random labeling of the deoxynucleotides, one may conclude that both turnover characteristics and composition mark low molecular weight DNA as functionally distinct from its high molecular weight counterpart.

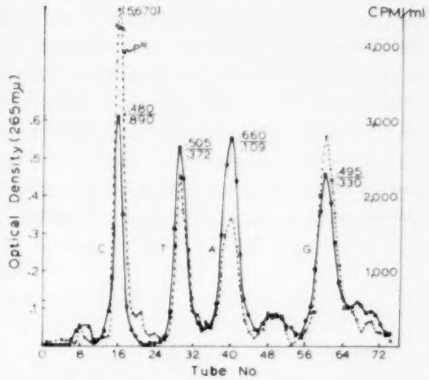

FIG. 2.—Elution pattern of low molecular weight DNA obtained from growing wheat roots. Only a small portion of the roots was labeled; the remainder of the low molecular weight DNA was obtained from wheat grown in a greenhouse. Optical densities at 260/280 mμ are given for the peak tubes. Symbols are the same as in Table 2. The fractionation system used was slightly modified from the original.[5] A 50 ml mixing flask was substituted, and the reservoir first filled with 0.2 *M* ammonium formate; the latter was replaced with 0.4 *M* ammonium formate after elution of thymidylate, and with 0.6 *M* after elution of adenylate. Each tube contained 3 ml of eluate.

To obviate the uncertainties implicit in the assumption of random deoxynucleotide labeling, a relatively large amount of low molecular weight DNA was prepared from wheat roots and added as carrier to a sample of labeled material. The deoxynucleotides were prepared and fractionated in the same way as those from corn roots. The results of such fractionation are shown in Figure 2. The parallel in curves of radioactivity and ultraviolet absorption is clearly apparent; so too is the preponderance of deoxycytidylate and deoxyguanylate residues over those of thymidylate and deoxyadenylate. The low molecular weight forms of DNA derived from corn and wheat roots are therefore similar.

To establish that the ultraviolet absorption was due to deoxyribonucleotides, each of the fractions was concentrated and assayed microbiologically for deoxy-

TABLE 1

RADIOACTIVITY OF DEOXYNUCLEOTIDES IN LOW MOLECULAR WEIGHT AND FIBROUS DNA

Nucleotide	cpm P^{32} × 25 Fibrous	Low m.w.
d-cytidylate	5,973	12,795
d-guanylate	5,645	13,326
d-thymidylate	9,598	10,420
d-adenylate	8,769	10,954
% GC	39	55

Corn seedlings were grown in the presence of P^{32}-phosphate for 24 hr. The two DNA fractions (fibrous and low molecular weight) were digested with deoxyribonuclease and phosphodiesterase using calf thymus DNA as carrier. The components were resolved as described for Fig. 2. The eluates were adsorbed on a Dowex-1 (Cl^-) column, washed with 1×10^{-4} *M* NH_4Cl (pH, 8), and eluted with 1.0 *M* NaCl.

riboside content.[6] With the exception of deoxycytidylate, a satisfactory correspondence was found between ultraviolet and microbiological data (see Table 2). To eliminate the possibility that traces of ribonucleotides were responsible for the observed radioactivities, a portion of each of the deoxynucleotide fractions was spotted on paper and resolved in an ethanol:ammonium acetate:borate mixture.[7] Only one ultraviolet-absorbing spot was observed for each eluate, and the R_f value was similar to that of a known sample of deoxynucleotide. The ultraviolet-absorbing spot and the remainder of the paper were extracted separately with 0.1 *N* HCl; radioactivity and deoxyriboside content were both determined. The deoxycytidylate and deoxyguanylate fractions, respectively, had 17 and 24% of their counts outside the spots; these values were taken as a measure of radioactive contamination. Thymidylate and deoxyadenylate showed negligible activity outside the spots, but, unlike the other two fractions, showed the presence of some deoxyribosidic material outside the observed spots. The results of these analyses together with various calculations are shown in Table 2.

TABLE 2

COMPOSITION AND RADIOACTIVITY OF DEOXYNUCLEOTIDE FRACTIONS

Fraction	Total cpm (corrected)	Total μmoles (u.v.)	Total μmoles (bioassay)	Total μmoles (corrected) bioassay)	S.A. (eluates)	S.A. (paper)
C	17,450	0.42	0.54	0.54	32,300	37,200
T	13,550	0.56	0.54	0.42	32,300	37,300
A	14,200	0.57	0.57	0.46	30,900	34,400
G	21,600	0.66	0.69	0.66	32,700	34,400
GC content (%)	58	49	51	58		

Total counts per minute in the pooled and concentrated eluates were corrected by multiplying the original counts in each by the percentage of counts localized in the deoxynucleotide spot on the paper chromatogram. The corrections were as follows: d-cytidylate (C), 83%; thymidylate (T), 93%; d-adenylate (A), 96%; d guanylate, 76%. The deoxyriboside content of each of the eluates was similarly corrected; the original and corrected values are recorded in the table. The specific activities (S.A.—cpm per μg DNA) of the pooled eluates and the paper spots were determined using the values of deoxynucleotide content measured by microbiological assay.

The main conclusion to be drawn from the above results is that the inference initially made respecting the presence of a metabolically labile form of DNA is correct. The near coincidence in values obtained by ultraviolet analysis, diphenylamine test, enzymatic digestion, and quantitative determination of the products by ultraviolet and microbiological analysis, leaves little room for alternative interpretations. It would be premature, however, to draw detailed conclusions about the composition of this form of DNA. Although enzymatic digestion of high molecular weight DNA yields the expected complement of deoxynucleotides, the minor irregularities in the elution pattern of low molecular weight DNA digests need not be attributed to experimental error. The possibility remains that distinctive minor components are present, and this may be related to the fact that only 80% of the DNA loaded on the column was recovered in the pooled fractions. Even so, the similarity in specific activities of the deoxynucleotide fractions, whether determined from the column eluates or from the paper spots, indicates that the turnover characteristics manifest in Figure 1 apply to the molecule as a whole.

Discussion.—In studies to be reported elsewhere, high molecular weight DNA was found to have the same composition irrespective of tissue of origin. No evidence for turnover of this DNA was obtained. Thus, with respect to all properties

studied, the high molecular weight form in higher plants behaves typically for genetic material. This does not appear to be true for the low molecular weight form. A number of reports have been published concerning the existence of DNA in chloroplasts[8] and of "nonfibrous" DNA in nuclei of calf thymus.[9] No satisfactory evidence exists for presuming a homology between the form studied here and those reported elsewhere. The fact that label can be almost entirely "chased" out of the molecule argues against its being a genetic determinant in the conventional sense. Its rapid formation in embryos induced to germinate suggests rather a physiological role. If so, the gross composition here reported may not be a constant characteristic, even though in roots of both wheat and corn the guanine-cytosine content appears to be greater than that of the high molecular weight form. At this stage of study the most important conclusion to be drawn is that a form of metabolically labile DNA exists.

Summary.—Growing tissues of higher plants have two forms of DNA. One of these has an average molecular weight of the order of 10^5 and is characterized by a relatively rapid rate of turnover. The composition of this DNA appears to differ from its high molecular weight counterpart.

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*INTERFERENCE WITH GLYCEROKINASE INDUCTION IN MUTANTS OF E. COLI ACCUMULATING GAL-1-P**

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The inhibitory effect of galactose on the growth of mutants of *E. coli* lacking gal-1-P-uridyl transferase has been established by the work of Kurahashi and Wahba[1] and Yarmolinsky *et al.*[2] The inhibition is very pronounced in a synthetic medium containing glycerol as sole carbon source, and the addition of glucose to the medium promptly relieves inhibition.[2] The growth inhibition is accompanied by the accumulation of gal-1-P in the cell.[3] Similar findings have been reported from this laboratory with another mutant of *E. coli* which lacks the enzyme UDPG synthetase.[4]

It is not clear how galactose causes growth inhibition in these mutants. The

favored hypothesis is that gal-1-6-P, formed presumably by phosphorylation of gal-1-P, inhibits phosphoglucomutase, *in vivo*,[5] in the same manner as it has been shown to act *in vitro*.[6] The level of UDPG is also markedly lowered in late stasis, and the addition of glucose to the medium restores the level to normal.[3, 7]

We wish to report an interesting *in vivo* effect of gal-1-P in galactose-sensitive mutants, which serves to underline the complex nature of the bacteriostatic effect of galactose in these mutants. The addition of galactose to the medium causes a marked lowering in the level of the inducible enzyme, glycerokinase, a key enzyme in the utilization of glycerol by *E. coli*.[8] This effect of galactose is confined to mutants known to accumulate gal-1-P and is more clearly elicited, when glycerol serves as a supplemental rather than as the sole source of carbon.

Materials and Methods.—The K-12 strain which was used stemmed from the collection of Drs. E. and J. Lederberg and was generously donated to us.

Galactose essentially free of glucose was obtained from Sigma Chemical Company, as were the following reagents: α-glycerophosphate dehydrogenase (2X crystallized 10 mg/ml suspension), α-galactose-1-phosphate (dipotassium salt), ATP (dipotassium salt), and o-NPG.

The growth conditions in general were those described previously[2] using tryptone broth (1%) or ammonia mineral medium[9] with 1% glycerol as sole carbon source.

Extracts for enzyme assays were prepared by suspension of the washed cells in 1–2 ml of 0.1 *M* phosphate buffer, pH 7.5 and subjected to sonication in a 10 KC Raytheon sonicator for 3 min. The sonicate was spun at 10,000 $\times$ *g* for 15 min, and the supernatant used immediately for enzyme assay.

Enzyme assays: Epimerase was assayed by the two-step procedure of Kalckar *et al.*[10] β-Galactosidase assay was carried out on sonicated extracts with o-NPG as substrate.[11] Glycerokinase assay was carried out essentially according to Kennedy.[12] The increment in O.D. at 366 mμ was followed in a Cary recording spectrophotometer. The reaction was linear for at least 5 min after addition of the substrate, glycerol. Proportionality to increasing enzyme concentration was observed up to a Δ O.D. of 0.02/min. One unit of the enzyme was taken as the amount of glycerokinase which caused an O.D. increment of 0.001/min.[12] Specific activity was expressed as units per mg protein.

Protein was estimated on the extracts by a microbiuret procedure[13] after precipitation with 0.35 *M* TCA.[11]

Results.—Glycerokinase levels in T^- cells grown in glycerol-galactose medium: Figure 1 presents the growth curve and the levels of glycerokinase, epimerase, and β-galactosidase at various time intervals following the addition of galactose to the transferaseless strain W 3104. Glycerol served as the sole carbon source in these experiments. Galactose ($10^{-2}M$) was added to the culture at an O.D. of 0.2. Epimerase activity was followed as a measure of the induction of the enzymes of galactose metabolism.

The following points, evident from the figure, are of interest: (1) epimerase is induced without an appreciable lag period following addition of galactose to the medium; (2) β-galactosidase induction shows some lag period, but the kinetics and the levels reached are not conspicuously different from what can be observed in other K-12 strains induced with galactose;[10] (3) the specific activity of glycerokinase shows an appreciable decrease one hr after the addition of galactose. There is no

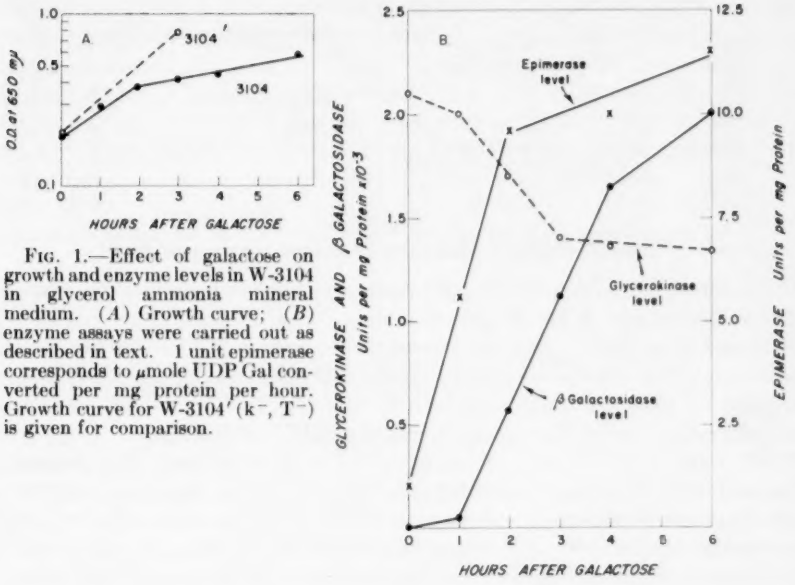

FIG. 1.—Effect of galactose on growth and enzyme levels in W-3104 in glycerol ammonia mineral medium. (*A*) Growth curve; (*B*) enzyme assays were carried out as described in text. 1 unit epimerase corresponds to μmole UDP Gal converted per mg protein per hour. Growth curve for W-3104′ (k^-, T^-) is given for comparison.

further drop in activity after the 3rd hr, by which time growth inhibition has set in. The drop in specific activity reflects a decreased synthesis of the enzyme as compared to the synthesis of the total protein.

In contrast, a galactose-resistant, kinaseless strain derived from 3104 showed no decrease in glycerokinase activity under comparable conditions, suggesting that galactose *per se* does not interfere with the induction of the kinase (Table 1).

TABLE 1

GLYCEROKINASE LEVELS IN GALACTOSE-RESISTANT KINASELESS STRAIN FROM 3104 IN GALACTOSE-GLYCEROL MEDIUM

Time after addition of galactose	O.D. 650 mμ at harvesting	Glycerokinase (units/mg protein × 10^3)	β-Galactosidase (units/mg protein* × 10^3)
0 hr	0.20	2.4	0.005
3 hr	0.78	3.6	2.01

* mμmoles o-NP liberated per min per mg protein.
Experimental conditions were the same as in Fig. 1.

Induction in broth medium: With a view to eliminate the possibility of growth inhibition as a contributory factor for the decrease in the activity of glycerokinase, the experiments were repeated using broth medium. In such a medium, galactose has little or no bacteriostatic effect. In these experiments, galactose ($10^{-2}M$) and glycerol (1 per cent final concentration) were both added at zero time to a broth culture not preinduced with either of these substrates. Galactose was withheld in control experiments. The cells were harvested in the log phase at an O.D. (Beckmann, 650 mμ) of 0.5. The results of such a study carried out with mutants of different phenotypes are presented in Table 2.

TABLE 2

Mutant	Phenotype	Glycerokinase (units/mg protein × 10^3) −Gal	+Gal
3100	$K^+T^+E^+$	1.33	1.10
3104	$K^+T^-E^+$	1.63	0.23
3104′	$K^-T^-E^+$	1.22	1.02
3096	$K^+T^-E^+$	0.81	0.07
4597	$K^+T^+E^+$ (UDPG synthetaseless)	0.95	0.10
3097	$K^+T^-E^+$	1.0	0.21
3097R	$K^+T^+E^+$	1.0	0.51

The cells were grown in 1% tryptone broth, 0.01 *M* $MgSO_4$—1% glycerol with or without galactose (10^{-2} *M*). The cells were not preinduced with either glycerol or galactose.

It is evident that galactose does not interfere with the induction of glycerokinase in the wild-type and in the kinaseless strains. The decrease in activity is very pronounced in mutants which are known to accumulate gal-1-P (80–95 per cent repression). In a revertant obtained from a transferaseless strain, there is some repression by galactose (approximately 50 %), but this is, in any case, much less than that seen in the transferase defectives or UDPG defectives.

Effect of extracts of T^- mutants and of gal-1-P on glycerokinase: The decrease in kinase activity in these experiments could be due to repression of enzyme formation or might represent inhibition of enzyme activity by gal-1-P or some other metabolite accumulated by the cell. To resolve this question, the effect of added gal-1-P and of extracts of galactose-grown transferaseless mutants on the activity of glycerokinase was studied. Again, a decrease in kinase activity could also be due to competition for the available ATP for phosphorylation of galactose and glycerol. The results presented in Tables 3 and 4 offer answers to these questions. (1) The addition of equimolar amounts of galactose and of glycerol to the glycerokinase assay medium has no effect on glycerokinase activity even when the ATP level is reduced to a third of the level used in the usual assay. (2) The addition of gal-1-P up to 1.2 μmoles to the assay mixture does not inhibit glycerokinase; nor is there an inhibition in activity when extracts of galactose-grown T^- cells are added to extracts obtained from cells grown in the absence of galactose.

TABLE 3

LACK OF INHIBITION OF GLYCEROKINASE BY GALACTOSE AND GAL-1-P

Additions to assay mixture	ATP (0.1 *M*)	Glycerokinase units*
(1) Nil	30 μl	37
(2) Galactose (0.1 *M*) 40 μl	30 μl	38
(3) Nil	10 μl	25
(4) Galactose (0.1 *M*)	10 μl	22
(5) Gal-1-P- (1.2 μmoles)	30 μl	36

* The values represent activity units in 10 μl of the enzyme extract.

Conditions of assay as in text. A concentrated extract of W-3104 grown in glycerol (1%)-galactose (10^{-2}*M*)-salts medium, having a specific activity of 0.46 glycerokinase units/mg protein, was used as source of glycerokinase. Reaction was started by the addition of 40 μl of 0.1 *M* glycerol after leveling off of the blank reaction.

TABLE 4

LACK OF INHIBITION OF GLYCEROKINASE BY EXTRACTS OF GALACTOSE-GROWN T^- CELLS

Enzyme source	Aliquot used for assay	Glycerokinase units	Sp. activity units/mg protein
(1) W-3104 grown in glycerol	10 μl	42	12.2
(2) W-3104 grown in glycerol + galactose	10 μl	4	2.0
(3) Mixed extracts of (1) and (2)	10 μl each	40	—

Assay mixture and other conditions are as described in text. The cells were grown in glycerol minimal medium with or without galactose (10^{-2}*M*).

Glycerokinase level in T⁻ cells grown in the presence of glucose: Cells of W 3104 preinduced in glycerol medium were distributed into two flasks containing medium of the same composition. After 2 hr at 37°, glucose at $10^{-3}M$ concentration was added to one of the flasks while the second flask served as control. The cells were harvested at an O.D. of 0.5 and the glycerokinase activity determined on the extracts. There was a sharp decrease in the specific activity of glycerokinase in the first case, as expected from the well-known "glucose effect" (Table 5).

TABLE 5

EFFECT OF GLUCOSE ON GLYCEROKINASE LEVEL IN W-3104

Addition	Glycerokinase (units/mg protein × 10^3)
Nil	1.22
Glucose	0.23

W-3104 grown in salts medium-glycerol. After 2 hr glucose ($10^{-3}M$) was added to one of the flasks and the other served as control. Cells harvested in the log phase and assayed for glycerokinase.

Discussion.—That gal-1-P represses glycerokinase formation is evident from the following observations:

(1) In transferaseless mutants growing in glycerol medium, the addition of galactose brings about a decrease in the specific activity of glycerokinase, the decrease becoming evident when the enzymes of galactose metabolism are well induced (Fig. 1). Since such a decrease is not observed in a T⁻ mutant which lacks galactokinase as well, the phosphorylation of galactose seems to be essential for the drop in glycerokinase activity. Since glycerol is the only usable carbon source, one might well consider the stasis as a result of a partial starvation (like a chemostat), although this needs further examination.

(2) The interference with the induction of glycerokinase is even more marked when the cells are growing in a broth medium, without preinduction in glycerol (Table 2). With mutants known to accumulate gal-1-P, the glycerokinase levels, in the presence of galactose, are about 10–20 per cent of the value observed with galactose omitted from the medium. Galactose does not interfere with glycerokinase induction either in the wild type, which is known to metabolize gal-1-P rapidly, or in K⁻ cells which have lost the phosphorylating mechanism for galactose.

(3) The decrease in glycerokinase activity is not due to inhibition of enzyme activity by gal-1-P or some other metabolite of galactose (Tables 3 and 4). The results are compatible with the assumption that gal-1-P inhibits formation of the enzyme.

The difference, noted in these experiments, in the response of the two inducible enzymes, β-galactosidase and glycerokinase, is of interest in view of the recent observations of McFall and Mandelstam[14] to the effect that inducible enzymes do not share a common metabolite repressor and that the repressor is identical with or closely related to the end-product of reaction catalyzed by the inducible enzyme. To explain the repression of glycerokinase by gal-1-P, the following possibilities may be considered.

The effect of gal-1-P could be indirect, through its adverse effect on phosphoglucomutase[5] and aldolase[15] resulting in the accumulation of glycolytic intermediates which might act as catabolite repressors.[16] Apart from this explanation, based on gal-1-P *per se*, one should consider the consequences of the generation of large

amounts of gal-1-P at the expense of ATP and the way this affects the levels of inorganic phosphate and especially of high energy phosphates and their dephosphorylated (partially or complete) products in the cell. Spyrides and Kalckar (unpublished observations), who looked into some of these questions, could not find any changes in ATP and ADP levels by the specific firefly assay method. However, the high energy phosphate pool includes mainly other nucleotides as well as intermediates like phosphoenol pyruvate and phosphoglycerate. Any increase in the levels of dephosphorylated products of the latter metabolites might result in the repression of glycerokinase.

Finally, it would be of interest to look into the effect of gal-1-P accumulation on the formation and/or activity of the permease system for glycerol.

Whatever the mechanism underlying the repressor effect of gal-1-P, it is obvious that the growth inhibitory effect of gal-1-P is more involved than hitherto assumed. The readiness with which glucose reverses stasis makes it probable that gal-1-P inhibits growth through the inhibition of formation and/or activity of essential enzymes involved in the utilization of a poor carbon source.

The paper was prepared during the author's stay at the Biochemistry Department of the Indian Institute of Science, Bangalore. He is pleased to record his indebtedness to Dr. H. M. Kalckar for his interest in the project and for many valuable suggestions which helped in the preparation of the paper. He is also thankful to Drs. K. Ebisuzaki and H. Nikaido for reviewing the manuscript.

The abbreviations used are: ADP = adenosine diphosphate; ATP = adenosine triphosphate; UDPG = uridine diphosphoglucose; G-1-P = glucose-1-phosphate; G-6-P = glucose-6-phosphate; Gal-1-P = α-galactose-1-phosphate; Gal-1,6-P = galactose-1,6-diphosphate; NAD = nicotinamide adenine dinucleotide; o-NPG = orthonitrophenyl galactoside; TCA = trichloroacetic acid; K^- = defective in galactokinase; T^- = defective in uridyl transferase.

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RELATIVISTIC SURFACE DYNAMICS OF AN ISOLATED WORLD TUBE OF PERFECT FLUID

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1. The dynamics of discontinuity surfaces in general relativity[1] has been used to examine the properties of the bounding surfaces of galaxies in the Hubble E-Series.[2, 3] In view of the fact that most previous relativistic galactic models have been based on the assumption that the momentum-energy tensor interior to a galaxy has a form characteristic of a perfect fluid, it seemed appropriate to examine the surface dynamics of an isolated world tube of perfect fluid. The results show that one obtains a unique representation of all limiting quantities through simple constructions in a subsidiary three-dimensional hyperbolic-normal metric space. It is also shown that the geodesic hypothesis can be invalidated only by internal dynamic processes. If the bounding surface is not known, a consistent procedure is given for the construction of the first and second fundamental forms and the boundary data such that a bounding surface is realizable in the underlying Einstein-Riemann space.

2. Let E be an Einstein-Riemann space whose metric structure is defined by the quadratic differential form

$$ds^2 = h_{AB}\, dx^A\, dx^B \ (A, B = 0, 1, 2, 3) \tag{2.1}$$

having signature -2. We denote by Σ a regular, timelike hypersurface in E which is defined by the parametric equations

$$x^A = f^A(u^0, u^1, u^2).$$

The normal vector to Σ, defined by

$$x_\alpha{}^A N_A = 0, \ (x^A{}_\alpha \overset{def}{=} \partial f^A(u)/\partial u^\alpha)$$

can be normalized by the requirement $N_A N^A = -1$. We shall also assume that any "time-slice" of Σ yields a closed two-surface. Let $a_{\alpha\beta}\, du^\alpha\, du^\beta$ denote the first fundamental form on Σ, then

$$a_{\alpha\beta} = \bar{h}_{AB} x_\alpha{}^A x_\beta{}^B$$

where the bar is used to denote evaluation on Σ. It is evident that the four vectors N^A, $x_\alpha{}^A$ for $\alpha = 0, 1, 2$ are linearly independent on Σ in E and it can be shown that

$$a^{\alpha\beta} x_\alpha{}^A x_\beta{}^B = \bar{h}^{AB} + N^A N^B; \tag{2.2}$$

also, the array of functions $a_{\alpha\beta}$ form a nonsingular symmetric form with signature -1. If we denote the coefficients of the second fundamental form by $b_{\alpha\beta}$, we have $b_{\alpha\beta} = b_{\beta\alpha}$ and

$$x_{\alpha;\beta}{}^{A} = b_{\alpha\beta}N^{A}, \tag{2.3}$$

$$N^{A}{}_{;\alpha} = b_{\alpha\beta}a^{\beta\gamma}x_{\gamma}{}^{A}. \tag{2.4}$$

Throughout this paper, Latin indices have the range 0, 1, 2, 3; Greek indices have the range 0, 1, 2; and the semicolon is used to denote covariant differentiation—the comma being reserved for the case of coordinate differentiation.

Let S_{AB} denote the jump strengths in the components of the momentum-energy tensor, that is,

$$S_{AB} = [T_{AB}] = T_{AB}(+) - T_{AB}(-), \tag{2.5}$$

where $T_{AB}(+)$ ($T_{AB}(-)$) denotes the limiting value of T_{AB} as Σ is approached from the exterior (interior). The results of reference 1 show that the functions

$$S_{\alpha\beta} \overset{\text{def}}{=} S_{AB}x_{\alpha}{}^{A}x_{\beta}{}^{B} \tag{2.6}$$

serve to determine the S_{AB}'s uniquely, this being a consequence of the existence requirements

$$S_{AB}N^{B} = 0. \tag{2.7}$$

In addition, the $S_{\alpha\beta}$'s must satisfy the fundamental system of equations

$$S_{\beta;\alpha}{}^{\alpha} = F_{\beta}, \tag{2.8}$$

$$S^{\alpha\beta}b_{\alpha\beta} + \chi = 0, \tag{2.9}$$

where

$$F_{\alpha} = F_{A}x_{\alpha}{}^{A}, \ \chi = F_{A}N^{A}, \tag{2.10}$$

and

$$F_{A} = [T_{A;C}{}^{B}]N^{C}N_{B}. \tag{2.11}$$

3. We assume that Σ is the boundary of a world tube of perfect fluid whose immediate neighborhood is empty. The components of the momentum-energy tensor interior to Σ are thus given by

$$T_{AB} = \mu W_{A}W_{B} - p(h_{AB} - W_{A}W_{B}) = \rho W_{A}W_{B} - ph_{AB}, \tag{3.1}$$

where

$$\rho = \mu + p, \ W_{A}W^{A} = 1, \tag{3.2}$$

while in the immediate neighborhood exterior to Σ all components of the momentum-energy tensor vanish. With a bar used to signify evaluation as Σ is approached from the interior, (3.1) yields

$$S_{AB} = -\bar{\mu}\bar{W}_{A}\bar{W}_{B} + \bar{p}(\bar{h}_{AB} - \bar{W}_{A}\bar{W}_{B}), \tag{3.3}$$

in view of the fact that $T_{AB} = 0$ immediately exterior to Σ. Since the functions S_{AB} must satisfy the four conditions (2.7), we have

$$0 = \bar{\mu}\bar{W}_A\bar{W}_BN^B - \bar{p}(N_A - \bar{W}_A\bar{W}_BN^B).$$

In view of the fact that S_{AB} admits $\bar{W}_A$ as an eigen vector, the above equations can be satisfied for $S_{AB} \neq 0$ if and only if

$$\bar{W}_AN^A = 0, \bar{p} = 0. \tag{3.4}$$

We thus have

$$S_{AB} = -\bar{\rho}\bar{W}_A\bar{W}_B \tag{3.5}$$

as the most general form of the jump strengths of a locally-isolated world tube of perfect fluid, the vanishing of $\bar{p}$ being used to obtain $\bar{\rho} = \bar{\mu}$.

4. In view of the first of (3.4) and the properties of the vectors $(N^A, x_\alpha{}^A)$, we have

$$\bar{W}^A = W^\alpha x_\alpha{}^A, \tag{4.1}$$

the quantities W_α being defined by

$$W_\alpha = W_Ax_\alpha{}^A.$$

Surface covariant differentiation of (4.1) and use of (2.3) gives

$$\bar{W}_{;\alpha}{}^A = W_{;\alpha}{}^\beta x_\beta{}^A + W^\beta b_{\beta\alpha}N^A. \tag{4.2}$$

Now,

$$\bar{W}_{;\alpha}{}^A = (\overline{W_{;B}{}^A})x_\alpha{}^B,$$

so that a contraction with $x_\gamma{}^Ca^{\alpha\gamma}$ together with (2.2) and (4.2) yields

$$(\overline{W_{;D}{}^A}) = (W_{;\alpha}{}^\beta x_\beta{}^Ax_\gamma{}^Ca^{\alpha\gamma} + W^\beta b_{\beta\alpha}a^{\alpha\gamma}x_\gamma{}^CN^A)\bar{h}_{CD} - (\overline{W_{;B}{}^A})N^BN_D. \tag{4.3}$$

When this result is combined with (3.4), we obtain the acceleration vector on Σ, namely,

$$(\overline{W_{;D}{}^AW_D}) = W^\beta{}_{;\alpha}W^\alpha x_\beta{}^A + W^\alpha W^\beta b_{\alpha\beta}N^A. \tag{4.4}$$

In a similar fashion we obtain

$$\overline{(\rho_{,C})} = \rho_{,\alpha}x_\beta{}^Ba^{\alpha\beta}\bar{h}_{BC} - \overline{(\rho_{,A})}N^AN_C, \tag{4.5}$$

$$\overline{(p_{,C})} = -\overline{(p_{,A})}N^AN_C; \tag{4.6}$$

the latter result stemming from the fact that $\bar{p} = 0$. We also have from (4.1) that

$$1 = \bar{W}^A\bar{W}^B\bar{h}_{AB} = W^\alpha W^\beta\bar{h}_{AB}x_\alpha{}^Ax_\beta{}^B = W^\alpha W^\beta a_{\alpha\beta} = W_\alpha W^\alpha.$$

Covariant differentiation of (3.1) yields

$$T_{A;C}{}^B = \rho_{,C}W_AW^B + \rho W_{A;C}W^B + \rho W_AW_{;C}{}^B - p_{,C}\delta_A{}^B.$$

Hence, on combining (2.11) and (3.4) and remembering the sign convention for jump strengths, we obtain

$$F_A = -\bar{\rho}\bar{W}^A(\overline{W_{;C}{}^B})N^CN_B + (\overline{p_{,C}})N^CN_A, \tag{4.7}$$

so that

$$F_\beta = -\bar{\rho}W_B(\overline{W_{;C}{}^B})N^C N_B, \tag{4.8}$$

$$\chi = -(\overline{p_{,C}})N^C. \tag{4.9}$$

We have thus obtained all significant differential expressions in terms of the geometry of Σ, together with surface and normal differential expressions.

5. We are now in a position to apply the fundamental system (2.8), (2.9). From (2.6) and (3.5) we have

$$S_\beta{}^\alpha = -\bar{\rho}W_\beta W^\alpha. \tag{5.1}$$

Hence, if we substitute (5.1) and (4.9) into (2.9), we arrive at

$$\bar{\rho}W^\alpha W^\beta b_{\alpha\beta} = -(\overline{p_{,C}})N^C. \tag{5.2}$$

Similarly, if we substitute (5.1) into (2.8) and use (4.9), we obtain

$$(\bar{\rho}W^\alpha)_{;\alpha}W_\beta + \bar{\rho}W_{\beta;\alpha}W^\alpha = \bar{\rho}W_\beta(\overline{W_{;C}{}^B})N^C N_B. \tag{5.3}$$

Contracting (5.3) with W^α and using the fact that $W_\alpha W^\alpha = 1$, gives us

$$(\bar{\rho}W^\alpha)_{;\alpha} = \bar{\rho}(\overline{W_{;C}{}^B})N_B N^C. \tag{5.4}$$

Thus, eliminating the common term between (5.3) and (5.4), we are led to

$$\bar{\rho}W_{\beta;\alpha}W^\alpha = 0. \tag{5.5}$$

In addition, with the aid of (4.3), we see that equation (5.4) is equivalent to the condition

$$\{\overline{(\rho W^A)_{;A}}\} = 0. \tag{5.6}$$

The vector field W_α thus defines geodesics in the three-dimensional hyperbolic-normal metric space Σ^* with metric differential form $a_{\alpha\beta}du^\alpha du^\beta = d\lambda^2$ and the quantity $\bar{\rho}$ is given by

$$\bar{\rho} = \rho_0 \exp\left\{\int (N_B N^C(\overline{W_{;C}{}^B}) - W^\alpha{}_{;\alpha})d\lambda\right\}. \tag{5.7}$$

If the motion is incompressible, that is $W_{;A}{}^A = 0$, we have $\bar{\rho} = \rho_0$ by (4.3).

6. Thomas[4] has pointed out that an isolated world tube of perfect fluid contains a fluid particle which describes a geodesic in E if $(\rho W^A)_{;A} = 0$. The above result, that this condition is rigorously satisfied on the boundary of such a world tube, indicates that there must be creation or annihilation of the flux ρW^A due to internal processes if a geodesic is not to result.

7. Under the customary assumption that the discontinuity problem is of second order in the h's, that is,

$$[h_{AB,}] = [h_{AB,C}] = 0,$$

jumps in the momentum-energy tensor imply jumps in the second coordinate derivatives of the h's. It has been shown[5] that there exists a symmetric tensor λ on Σ such that

$$[h_{AB,CD}] = \lambda_{AB}N_C N_D. \tag{7.1}$$

If the Einstein field equations are to be solvable, it may be shown[1] that we must have

$$\lambda_{\alpha\beta} = \kappa(2S_{\alpha\beta} - Sa_{\alpha\beta}), \tag{7.2}$$

where

$$\lambda_{\alpha\beta} = \lambda_{AB}x_\alpha{}^A x_\beta{}^B. \tag{7.3}$$

The inversion of (7.3), as given by equation (3.17) of reference 1, then determines the jump strengths λ_{AB} of the metric field to the extent that such determinations may be effected. For the case at hand, (5.1) and (7.2) give

$$\lambda_{\alpha\beta} = \kappa\bar{\rho}(2W_\alpha W_\beta - a_{\alpha\beta}). \tag{7.4}$$

8. If we assume that the hypersurface Σ is known, the above results give a complete statement of the boundary data for a world tube of perfect fluid. One takes a timelike geodesic in the metric space Σ^* with unit tangent vector (W^α). The limiting values of the velocity field are then uniquely determined by

$$\bar{W}^A = W^\alpha x_\alpha{}^A, \tag{8.1}$$

and the quantities $\bar{\rho}$ and $\bar{p}$ are given by (5.7) and $\bar{p} = 0$. The jump strengths of the metric field are then given by (7.4).

9. Suppose now that the equation for Σ is not known. In this case, the results of the previous paragraph still give us the appropriate boundary data, but the quantities $a_{\alpha\beta}$, $b_{\alpha\beta}$, and $x_\alpha{}^A$ are unknown. Since the Gauss-Cadazzi equations can be shown to be valid for second-order problems,[5] we can still obtain a complete determination of the boundary data if we can find consistent means of determining the quantities $a_{\alpha\beta}$. It has been shown[1] that the differential system (2.8) can be integrated in the space Σ^* under rather general circumstances. The results are

$$S_{\alpha\beta} = \textcircled{H}(K_{\alpha\beta} - \frac{1}{2}(K + \Pi)a_{\alpha\beta}) + Q_{\alpha\beta}, \tag{9.1}$$

where $K_{\alpha\beta}$ are the components of the Ricci tensor of Σ^*, K is the scalar curvature of Σ^*, $\textcircled{H}$ and Π are constants, and $Q_{\alpha\beta}$ are the components of a symmetric tensor such that

$$Q_{\alpha;\beta}{}^\beta = F_\alpha. \tag{9.2}$$

For the case of a perfect fluid, we therefore have

$$-\bar{\rho}W_\alpha W_\beta = \eta(K_{\alpha\beta} - \frac{1}{2}(K + \Pi)a_{\alpha\beta}) + Q_{\alpha\beta} \tag{9.3}$$

and

$$Q_{\alpha;\beta}{}^\beta = -\bar{\rho}W_\alpha\phi, \tag{9.4}$$

where

$$\phi = (\overline{W_{;B}{}^A})N^B N_A. \tag{9.5}$$

Hence, for any particular solution of (9.4), the system (9.3) becomes a differential system for the determination of the quantities $a_{\alpha\beta}$. Any system of a's determined

in this fashion can be combined with the Gauss-Cadazzi equations and the previous results to yield the remaining unknowns $b_{\alpha\beta}$ and $x_\alpha{}^A$. If one proceeds in this manner, cognizance of certain intrinsic properties of the bounding surface Σ can be included in a straightforward manner. For instance, if it is known that the hypersurface Σ admits a group of motions, this fact can be combined with (9.3) so that the resulting a's will exhibit such properties (see sec. 6 of ref. 1 for a particular example). In the particularly simple case in which $\phi = 0$, we can take $Q_{\alpha\beta} = 0$. The system (9.3) then shows that the space Σ^* is a three-dimensional analogue of an Einstein-Riemann space with incoherent matter.

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THE ROLE OF SODIUM IONS IN THE ACTIVATION OF ELECTROPHORUS ELECTRIC ORGAN ADENOSINE TRIPHOSPHATASE

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A phosphorylated enzyme intermediate has been proposed to explain the exchange kinetics of the Na^+-activated adenosine triphosphatase of crab nerve.[1] A reaction with similar activation characteristics[2] is catalyzed by a small particle fraction derived from electric organ tissue of *Electrophorus electricus*. We have recently reported that these particles will incorporate radioactive phosphate upon brief incubation with ATP^{32}.[3] This and related observations are presented in greater detail in the present communication, together with some observations on the possible correspondence of the mechanisms of ATP hydrolysis and sodium ion transport.

Materials and Methods.—(a) *Preparation of particle fraction containing sodium-activated ATPase:* Electric organ tissue is dissected from freshly killed eels, minced with scissors, and disintegrated in a stainless steel blendor in 10 volumes of 0.05 *M* Tris buffer, pH 7.5. After straining through gauze, the homogenate is centrifuged 20 min at 9,000 × *g*. The precipitate is resuspended in 10 volumes of buffer and sonicated for 3 one-min periods, maintaining the temperature of the suspension at 6°C; after centrifuging as above, the combined supernatant is centrifuged for one hr at 50,000 × *g*. The sonication and extraction of the 9,000 × *g* precipitate may be repeated one or more times to increase the yield.

The 50,000 × *g* precipitate containing the ATPase is resuspended to a protein concentration of approximately 10 mg/ml in 0.1 *M* Tris pH 7.5, divided into small aliquots, and rapidly frozen by immersion in dry ice-acetone. The quick-frozen particles can be stored for several months at −20°C with little loss of ATPase activity.

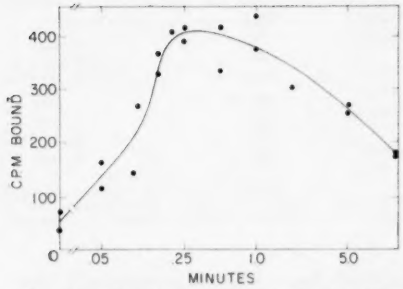

FIG. 1.—Kinetics of ATP^{32}-binding by ATPase fraction. Reaction mixture is described in Table 1 with the following exceptions: 0.003 *M* $MgCl_2$; 0.001 *M* ouabain; 200,000 cpm ATP^{32} (1 mμmole) was added together with 0.5 μmole P to initiate the binding reaction.

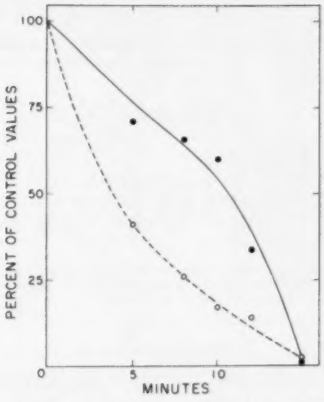

FIG. 2.—Rate of heat inactivation of ATP^{32}-binding activity and of ATPase activity at 47°C. Incubation mixture as for Fig. 1. Solid circles are P^{32}-binding data; open circles indicate relative rates of hydrolysis.

(b) *Preparation and assay of ATP^{32}:* The ATP^{32} used in this study was prepared from carrier-free P^{32} (Oak Ridge) and pyridine ADP according to the procedure of Lowenstein.[4] The nucleotide was isolated according to a modification of the method of Morell *et al.*[5] The ATP concentration was determined using firefly luciferinase.[6] The extent of γ-labeling was determined by hydrolysis with ATPase and separation of the radioactive orthophosphate by thin layer chromatography.[7]

(c) *P^{32} incorporation experiments:* The enzyme was incubated with ATP^{32} in small Pyrex tubes (3 × 60 mm) in an ice bath. Additions were made with specially designed Lang-Levy pipettes and mixed by "buzzing."[8] After 10% TCA denaturation and centrifugation at 1,500 × *g* for 10 min in a refrigerated centrifuge, the supernatant was removed. The precipitate was resuspended in cold washing solution (5% TCA containing 0.05 *M* Na_2ATP and 0.05 *M* KH_2PO_4) and again centrifuged. After 3–4 washings the radioactivity remaining in the final precipitate was counted by inserting the tube in the well of a plastic phosphor block adapted to the Packard Tri-Carb scintillation spectrometer.

Results and Discussion.—(a) *Characteristics of the incorporation of radioactive phosphate from ATP into the particles:* Maximal incorporation of P^{32} is attained within 15–30 seconds at 0°C (Fig. 1). This bound radioactivity is stable to repeated washing with cold trichloroacetic acid containing orthophosphate and ATP. Only slight diminution of the labeling results when orthophosphate is present in 1,000-fold excess of the ATP (Table 1). This phosphorylation is heat-sensitive (Fig. 2). The level of phosphorylation is increased in the presence of Mg^{++} (Table 2*A*). At the optimal Mg^{++} concentration there is a further increase

TABLE 1

Additions	Cpm (15′-0)
0.1 mμmole ATP^{32} (72,000 cpm)	317
0.1 mμmole ATP^{32} + 0.16 μmole Tris PO_4	274
0.1 mμmole ATP^{32} + 0.16 μmole Tris ATP	1

Comparison of effects of dilution of ATP^{32} by orthophosphate and ATP on amount of P^{32} incorporated: The incubation volume of 70 μl contained 200 μg ATPase protein; 5.4×10^{-3} *M* $MgCl_2$; 1.8×10^{-3} *M* ouabain; 0.06 *M* Tris-HCl, pH 7.5. The radioactive ATP (76% γ-labeled) was added simultaneously with the nonradioactive ATP or orthophosphate. Incubations were terminated after 15 sec at 0° by adding 8 μl 50% TCA. In the case of zero time controls, the order of addition of TCA and ATP^{32} was reversed. The acid precipitates were washed 3 times with a solution containing 5% TCA, 0.05 *M* Na_2ATP and 0.05 *M* KH_2PO_4 before counting.

TABLE 2A

EFFECT OF CATIONS ON PHOSPHORYLATION OF ELECTRIC ORGAN PARTICLES

Additions	Bound radioactivity cpm (25″-0)
None	92
Mg^{++}, 0.003 M	145
Mg^{++}, 0.003 M + Na^+, 0.06 M	345
Mg^{++}, 0.003 M + K^+, 0.025 M	114
Mg^{++}, 0.003 M + Na^+, 0.06 M + K^+, 0.025 M	264
EDTA, 0.002 M	94

The incubation volume of 50 μl contained enzyme, the indicated concentration of cations as chlorides, 0.02 M Tris phosphate pH 7.5, and 13 mμmoles ATP^{32}, 6×10^5 cpm.

TABLE 2B

EFFECT OF HIGH CONCENTRATIONS OF NaCl ON PHOSPHORYLATION OF ELECTRIC ORGAN PARTICLES

NaCl	Bound radioactivity cpm (25″-0)
0.06 M	833
0.24	1,449
0.50	3,315
1.00	4,360

Conditions as in 2A except that 0.003 M $MgCl_2$ was present in all tubes. The 13 mμmoles of ATP contained 1.4×10^5 cpm.

in extent of phosphorylation in the presence of Na^+. K^+ superimposed upon Mg^{++} and Na^+ reduces the amount of labeling. The extent of labeling as a function of ATP concentration follows Michaelis kinetics in the presence of Mg^{++} and ouabain (Fig. 3). The apparent K_m under these conditions is 1.2×10^{-4} M. This compares with $K_m = 8 \times 10^{-5}$ M for hydrolysis.

(b) *Characteristics of the isotope exchange reaction:* The electric organ ATPase is similar to that of crab nerve[1] in catalyzing the equilibration of labeled ADP with ATP (Table 3). At concentrations which maximally activate the hydrolysis,

TABLE 3

I		II	II/(I + II)
(a) Kinase exchange C^{14}-ADP	→	ATP	0.170
(b) Adenylate kinase C^{14}-AMP	→	ATP + ADP	<0.003
(c) Adenylate deaminase C^{14}-AMP	→	IMP	<0.003
(d) Adenylate cyclase C^{14}-ATP	→	3′, 5′-AMP	<0.001

Nucleotide interconversion reactions catalyzed by electric organ particles: All incubations were of 20 min duration at 26°C with 2.5 μg enzyme; 3 μmoles Tris-HCl, pH 7.2; 0.1 μmole $MgCl_2$; 45 μl total volume. (a) 0.3 μmole Tris ATP; 0.003 μmole C^{14}-ADP. (b) 0.28 μmole C^{14}-AMP; 0.36 μmole ADP; 0.36 μmole ATP. (c) 0.1 μmole C^{14}-AMP; 0.15 μmole IMP. (d) 0.12 μmole C^{14}-ATP, 0.2 μmole 3′, 5′-AMP.

After deproteinization with 4% formic acid in methanol, the nucleotides were separated by thin layer chromatography[7] and counted by liquid scintillation technique.

Na^+ and K^+ have no appreciable effect on the exchange rate. P^{32} does not measurably exchange into ATP.

(c) *Comparison of the properties of the hydrolytic, exchange, and particle phosphorylation reactions:* Since the particulate preparation may contain enzyme activities other than the ATPase, it is important to establish the extent to which the three reactions under study may be facets of the same catalytic entity. There is no detectable hydrolysis of ATP by this preparation unless both Mg^{++} and Na^+ are present.[2] Adenylate kinase is not detectable as measured by the complete absence of incorporation of C^{14}-AMP into ADP (Table 3), and hexokinase is not detectable by spectrophotometric assay.

However, other kinases may contribute to the observed phosphorylation and exchange reactions. The initial rate of equilibration of C^{14}-ADP with ATP may be equated with the rate of phosphorylation of the enzyme protein: ATP + E $\rightleftarrows$ ADP + E − P. Under conditions of suboptimal Mg^{++}, this step can be made

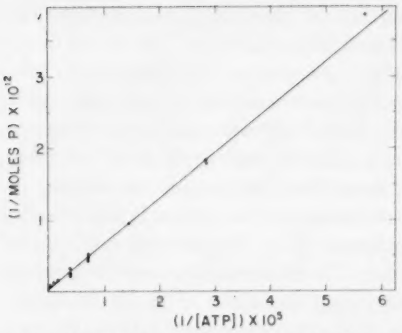

FIG. 3.—Effect of ATP^{32} concentration on amount of radioactive phosphate bound. ATPase fraction equivalent to 0.30 mg protein was incubated in 54 μl containing 0.01 *M* Tris phosphate, pH 7.2; 0.003 *M* $MgCl_2$; 0.001 *M* ouabain. Samples denatured and washed as described in Table 1.

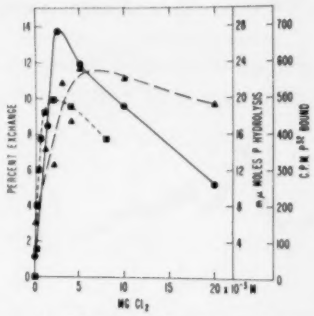

FIG. 4.—Mg^{++} activation. (●———●) ATPase activity at optimal Na^+ and K^+ concentrations; (■———■) C^{14}-ADP exchange into ATP, no alkali metal ions; (Δ———Δ) P^{32} incorporation from ATP^{32} into particles, no alkali metal ions.

rate-limiting in the ATPase reaction. Rate of exchange and rate of hydrolysis will be direct and inverse functions, respectively, of the ADP concentration, so that a simple quantitative comparison is not valid. However, under our assay conditions, the measured rates of isotope equilibration and of hydrolysis are of the same order of magnitude. The ATPase reaction must therefore account for a large part of the observed exchange reaction.

All three reactions, while requiring Mg^{++}, are inhibited at higher concentrations. The optimal Mg^{++} concentrations are similar for the three reactions (Fig. 4).

The small effects of sodium and potassium ions on the exchange reaction led Skou to ascribe their principal role in the ATPase mechanism to an activation of the hydrolysis of the phosphorylated enzyme.[1] The effect of K^+ on particle phosphorylation is consistent with this (Table 2*A*). However, the increment of phosphorylation in the presence of Na^+ constitutes a qualitative distinction in the roles of these two cations. These results are in agreement with those observed for the labeling of kidney particles by ATP^{32}.[9] The opposing effects of Na^+ and K^+ upon the level of phosphorylation, when considered in the light of the mutual competition of Na^+ and K^+ in the hydrolytic reaction,[10] led to the expectation that very high concentrations of Na^+

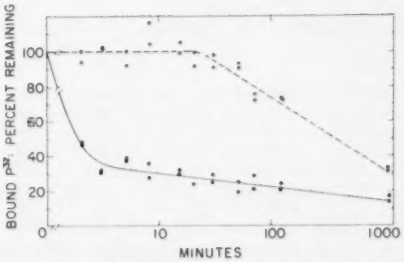

FIG. 5.—Release of bound radioactivity by treatment with pepsin. P^{32}-labeled denatured ATPase fraction was prepared under the conditions of Table 1 except on a larger scale: 7 mg protein was incubated in 1.0 ml. The denatured preparation was rehomogenized after 8 washes. One half was taken for incubation in 1.0 ml 0.01 *N* HCl containing 10 mg pepsin. The remaining half was incubated in 0.01 *N* HCl as a control. Incubation was at 25°C. Aliquots were withdrawn at the indicated intervals, added to small tubes containing 1/10 volume 50% TCA, and counted before and after removal of the TCA soluble supernatant.

might increase the extent of labeling still further by blocking dephosphorylation at the K^+ site. This effect was obtained (Table 2*B*).

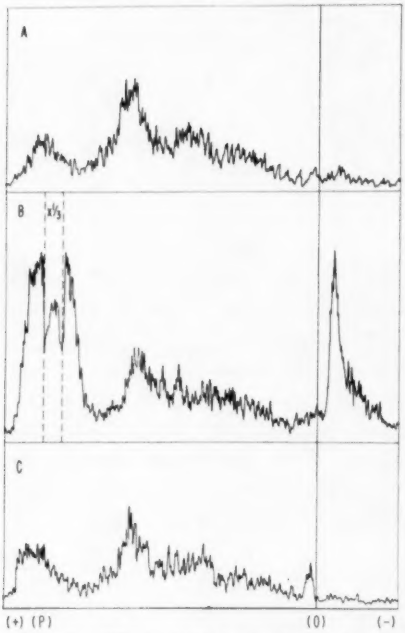

FIG. 6.—Effect of Na^+ on electrophoretic pattern of radioactive fragments released by pepsin. Approximately 1 mg of particles were labeled by incubating at pH 7.5 in 0.02 *M* Tris HCl containing 44 mμmoles ATP^{32} (330,000 cpm/mμmole) and 3×10^{-3} *M* $MgCl_2$. In addition, *B* contained 1.0 *M* NaCl, and *C* contained 1.0 *M* LiCl. Incubation was for 25 seconds at 0°C. The washed, acid-denatured precipitate was incubated with 1% pepsin, pH 2, for 10 min at 25°C. After precipitation with 20% TCA, the supernatant was extracted with 5 volumes of ether to remove the TCA before lyophilizing. Electrophoresis conditions: pH 3.5, 0.05 *M* ammonium formate; 5,000 volts; 90 min; 12°C. (*P*) indicates mobility of orthophosphate.

(d) *Enzymatic degradation of P^{32}-labeled particles:* Although the particle-bound P^{32} is stable to repeated washings with cold acid, brief alkaline treatment or prolonged incubation at room temperature releases most of the radioactivity in the form of orthophosphate. Brief incubation of P^{32}-labeled particles with pepsin in 0.01 *N* HCl also results in the release of about 70 per cent of the P^{32} in acid-soluble form (Fig. 5). However, only a small fraction of this is in the form of orthophosphate in the peptic digest derived from Mg^{++}-activated particles (Fig. 6*A*). At pH 3.5 the electrophoretic pattern of this material consists of three major peaks in addition to orthophosphate, all on the anodal side. The corresponding pattern from particles labeled in the presence of both Mg^{++} and Na^+ consists of the same series of peaks and an additional peak on the cathodal side (Fig. 6*B*). Moreover, the orthophosphate peak is greatly increased. When additional precautions are taken to reduce the exposure to acid, the cathodal peak is increased relative to the orthophosphate (Fig. 7).

(e) *Possible mechanism for the sodium-activated ATPase:* The foregoing observations may be reconciled by the following proposed mechanism:

$$(1)\quad \mathrm{ATP} + XEY \overset{Mg^{++}}{\rightleftharpoons} \mathrm{ADP} + \mathrm{P} \sim XEY$$

$$(2)\quad \mathrm{P} \sim XEY \overset{Na^{+}}{\rightleftharpoons} XEY - \mathrm{P}$$

$$(3)\quad XEY - \mathrm{P} + \mathrm{H_2O} \overset{K^{+}}{\rightleftharpoons} XEY + \mathrm{P}$$

$$(4)\quad \mathrm{ATP} + \mathrm{H_2O} \xrightarrow{Mg^{++},\ Na^{+},\ K^{+}} \mathrm{ADP} + \mathrm{P}$$

The particles are considered to contain three distinct catalytic activities which constitute the ATPase reaction in sum: a Mg^{++}-activated kinase, a Na^+-activated transferase, and a K^+-activated phosphatase. This scheme is not meant to specify the number of protein subunits which may be associated with these reactions, nor does it rule out the participation of intermediates other than phosphorylated proteins. However, in several attempts, we have been unable to remove any appreciable fraction of the particle-bound P^{32} by extraction with ethanol, butanol, or chloroform-methanol.

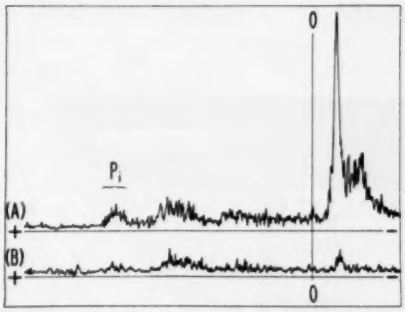

FIG. 7.—Electrophoretic pattern of radioactive acid-soluble fragments after pepsin. Labeling in the presence of 1.0 *M* NaCl and 0.003 *M* $MgCl^2$ (*A*) and 0.003 *M* $MgCl^2$ (*B*). Conditions as in Fig. 6, except that the exposure time to TCA was minimized by extracting the peptic digest with ether immediately after addition of TCA.

It seems reasonable to associate the sodium-augmented component of Figure 7 with the $Y - P$ component of the enzyme. The increment of P_i^{32} labeling in the presence of high $Na^+ + Mg^{++}$ may be 5–10-fold of that attained with Mg^{++} alone (Table 2; Fig. 7). For this reason, the structure of the ATPase unit may be better represented as XEY_n. As discussed below, the theoretical requirements for a cation-selective transport mechanism make it attractive to consider that a *series* of Na^+-dependent transfer reactions may intervene between the kinase and phosphatase steps:

$$(-Y_j - P) + (-Y_k) \xrightarrow{Na^+} (-Y_j) + (-Y_k - P).$$

(f) *Relation of the proposed mechanism to a scheme for active transport:* Any hypothetical scheme for active transport must provide (1) a sufficient source of energy, (2) a mechanism for countergradient translation, and (3) the appropriate specificity. In addition, it must be reconciled with whatever homeostatic controls and pharmacologic sensitivities have been demonstrated to apply to the physiological process.

The proposed elemental reactions of the Electrophorus ATPase can be incorporated into a mechanism for active transport of sodium ions which satisfies most of the preceding criteria.

(1) The energy requirements of active sodium transport have received considerable study,[11–14] but are still in doubt. The upper limit for transfer of sodium by any carrier system consisting of esterified phosphate is 2 Na^+ per P. If the chemical potential of the system decreases in the successive configurations ($P \sim XEY$) → ($XEY - P$) → (XEY, P), the energy released in these transformations may be utilized in the transport process.

(2) An orientation of the kinase, transferase, and phosphatase reactions, in that order, from the inner to the outer surface of the cell membrane (Fig. 8) would result in the translocation of phosphate from within the cell to the outer region of the cell membrane. If sodium is specified as the counterion in this process, its

transport would be the coupled, energy-consuming element of the sequence of reactions.

(3) The specificity of the transferase for sodium ions is therefore the essential factor in obtaining work from the system. In the model under consideration, phosphate is visualized as migrating down a free-energy gradient which drives it across the cell membrane through an environment which selects for sodium as the counterion. That a sufficient degree of selectivity is operant in the particles is evidenced by the activation kinetics of ATP hydrolysis. Eisenman has presented an excellent analysis of the factors which enter into the cation specificity of fixed anionic sites.[15] Some experimental support for the concept of a sodium-catalyzed phosphate migration may be derived from the observations of Taborsky[16] on the metal-catalyzed migration of phosphate in phosvitin.

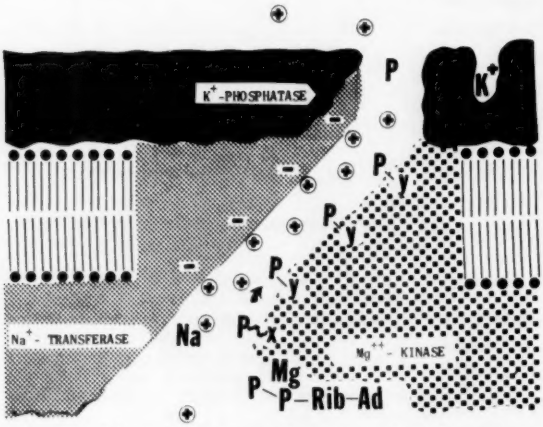

FIG. 8.—Schematic representation of an oriented membrane ATPase. Transphosphorylation from the primary phosphorylation site (P∼X) to P—Y can only occur in the presence of sodium ions, ⊕. Other counterions are rejected by the environment of fixed charges within the membrane.

In common with that of Hokin and Hokin,[17] the present model does not explicitly account for the return of phosphate into the cell. One function of the fixed negative charges at the external cell surface, which are believed to consist chiefly of sialic acids[18, 19] may be to screen the escape of phosphate ions. A back diffusion of phosphate set up in this manner, if channeled through a potassium-selective environment, could account for the coupled transport of K^+.[20, 21]

Summary.—Labeling of electrophorus electric organ Na^+-ATPase particles by ATP^{32} is stimulated by Mg^{++}. The sites available for phosphorylation are increased severalfold when Na^+ is also present. K^+ decreases labeling and Li^+ is without effect. Brief peptic digestion of "Mg^{++} sites" releases 3 major radioactive fragments, in addition to orthophosphate, which migrate anodally at pH 3.5. The electrophoretic pattern from "Mg^{++} + Na^+ sites" contains an additional slowly migrating cathodal component which readily breaks down to release orthophosphate.

Since Mg^{++}, but not Na^+, activates the ADP-ATP exchange reaction, Na^+ sites are evidently phosphorylated secondarily to the Mg^{++} sites. These observations suggest that Na^+ transport may be a consequence of oriented transphosphorylations across the cell membrane through an environment which selects for Na^+ as the counterion.

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*DEMONSTRATION OF TWO POPULATIONS OF CELLS IN THE HUMAN FEMALE HETEROZYGOUS FOR GLUCOSE-6-PHOSPHATE DEHYDROGENASE VARIANTS**

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In 1932, Muller coined the term "dosage compensation"[1] to account for the equality of phenotypic expression in males and females for most genes located on the X chromosome. Over the years several explanatory theories for this phenomenon have been considered, and these have been recently reviewed by Stern[2] and by McKusick.[3]

In 1961, a unique hypothesis was developed by Lyon[4] and by Russell.[5] The hypothesis, often referred to as the "Lyon Hypothesis," proposes that in each somatic cell of the female, one of the two X chromosomes is genetically inactive. The inactivation must occur early in development, and it is a matter of chance whether the maternal or paternal X is inactivated. Once an X chromosome is inactivated in a developing cell, all progeny of that cell presumably maintain the same inactive X.

Examination of single cells in a female who is heterozygous for an X-linked gene(s) with a measurable effect would provide direct evidence bearing on the hypothesis. In such a case, the female would be expected to produce a mosaic of X chromosome activity. In some of her cells, one allele would be active; in the remainder, the other allele would function.

The enzyme, glucose-6-phosphate dehydrogenase (G-6-PD), provides both a quantitative and a qualitative tool for testing the hypothesis. It has been shown that in a Caucasian male whose erythrocytes have deficient G-6-PD activity cultured skin cells are also clearly deficient.[6] Our own studies have confirmed this and have shown the ranges of activities for skin cells of the various genotypes.[7] If the "Lyon Hypothesis" holds, the heterozygous Caucasian female should produce two quantitatively different cell populations, one with G-6-PD activity in the mutant range, and one with activity in the normal range.

Starch gel electrophoresis has demonstrated qualitative variants of erythrocyte and leucocyte G-6-PD in the American Negro population, but not among Caucasians.[8, 9] Negro males can have a fast G-6-PD band, A, or a slow one, B. Females can have A, B, or both A and B. These studies have proved that the electrophoretic variants are inherited, and that the responsible genetic locus is on the X chromosome. Thus, if the "Lyon Hypothesis" applies, the female who is heterozygous for the two electrophoretic variants should also be a mosaic—some of her cells producing A type G-6-PD, some the B type, but none producing both.

The quantitative and qualitative variants are genetically related, but the precise relationship is not clear. With rare exceptions, among Negroes, all males with erythrocyte G-6-PD deficiency have the A electrophoretic variant. However, not all males with A variant are enzyme-deficient. To date, all Caucasians have the B electrophoretic band.

This paper reports the results of quantitative and qualitative analyses of clones derived from single cells from females heterozygous for the G-6-PD variants.

Materials and Methods.—Individuals studied: Skin biopsies from 17 Caucasian males and females were obtained in Sardinia, and these were studied for G-6-PD deficiency.[7] The genotype of each individual was designated in a previous family analysis.[10]

The Negro females presumed to be heterozygous for the qualitative electrophoretic variants were selected on the basis of appearance of two electrophoretic G-6-PD bands in extracts of cultured cells. Four of the six women were also studied for erythrocyte G-6-PD deficiency[10] and are heterozygous for the enzyme deficiency as well. Two of the women with the AB phenotype were found among Negro female employees at Sinai hospital.

Experimental procedures: (1) *Skin biopsies:* Biopsies were obtained without anesthesia using a high-speed electric rotating biopsy punch.[11] A core drill of 3 mm in diameter was employed. (2) *Culture technique:* The cores of skin were minced into 20 or 30 tiny fragments and cultured in Eagle's "Minimum Essential Medium" with 20% human AB or B serum and 5% beef embryo extract ultrafiltrate.[12] Plastic culture dishes were used, and the tissue was incubated at 37°C in an atmosphere of 5% CO_2 in air. Clones were developed from single cell platings of cultured cells according to the techniques described by Puck *et al.*[13] (3) *Assay of G-6-PD activity:* Suspensions of cultured cells were disrupted by sonication, and enzyme activities were measured by a modification of the method of Kornberg and Horecker.[14] A Beckman DU spectrophotometer, equipped with a Gilford optical density converter, model 220, was used. G-6-PD activities are expressed as Δ O. D. per mg of protein per hr. (4) *Electrophoresis:* Sonicates of cultured cells were subjected to starch gel electrophoresis, using the Smithies technique.[15] The continuous buffer system of Kirkman[8] was used, substituting 0.02 *M* sodium barbital for this, and the gels were developed after 15–16 hr using the procedure described by Boyer *et al.*[9]

Results.—Quantitative study: In Table 1, G-6-PD activities are given for two proved heterozygous Caucasian women. Four clones were analyzed from each, and in each case one clone was clearly in the deficient range and three in the normal range. The established ranges of G-6-PD activity in skin cell culture for normal and deficient Caucasians are given below the table.[7]

TABLE 1

CLONE RESULTS—HETEROZYGOUS CAUCASIAN FEMALES (G-6-PD ACTIVITY/MG PROTEIN)

Individual	Original activity*	Clone number	Clone activity
Sardinian #3	6.3	1	12.9
		3	8.7
		5	9.0
		2	1.2
Sardinian #5	2.4	13	2.7
		2	9.7
		15	9.1
		16	10.3

Ranges of G-6-PD activity for cultured Caucasian skin cells

Normal (males and females): 5.2–16.2

Deficient { Hemizygous males / Homozygous females : 0.7–3.4

* Refers to the activity of the cultured cell strain prior to cloning.

In Table 2, original and clonal G-6-PD activities are compared for various genotypes. It is quite evident that deficient individuals produce clones with deficient enzyme activities, and clones derived from normal individuals have normal enzyme activities.

TABLE 2

CLONE RESULTS—NORMAL AND DEFICIENT CAUCASIANS

Individual	Sex	Genotype	Original activity	Clone number	Clone activity
Sardinian #4	Male	Hemizygous mutant	1.8	1	1.3
				2	1.2
				5	2.1
Sardinian #13	Female	Homozygous mutant	2.3	4	1.6
Sardinian #12	Male	Hemizygous normal	10.4	1	13.7
Sardinian #14	Female	Homozygous normal	11.1	4	11.3

Qualitative (electrophoretic) study: Clones have been developed from single cell platings from six Negro women found to have the two G-6-PD electrophoretic bands; that is, they are all presumed heterozygotes for the electrophoretic variants. With no exceptions, the clones have yielded only single bands, some of which are A and some B. Table 3 shows the number of A and B clones for each heterozygote. Figure 1 depicts two heterozygous females (double bands), and three individuals with single bands, 2 A's and 1 B. Figure 2 shows an original double band with nine clones beside it, 3 A's and 6 B's.

Discussion.—Both the quantitative and qualitative clonal data show that, with respect to the G-6-PD locus, there are indeed two distinct populations of cells in the heterozygous female. This is direct evidence in favor of the "Lyon Hypothesis"—in each single cell of the female only one G-6-PD locus is operative. Thus,

TABLE 3

RESULTS OF STARCH GEL ELECTROPHORESIS OF CLONES FROM WOMEN WHO POSSESS THE AB ELECTROPHORETIC G-6-PD PHENOTYPE

	Mrs. Bi.	Mrs. Mi.	Mrs. Wi.	Mrs. Bo.	Mrs. De.	Mrs. Ha.
Type A	8	1	0	8	7	0
Type B	2	8	8	0	7	5
Type AB	0	0	0	0	0	0
Total number of clones	10	9	8	8	14	5

FIG. 1.—Starch gel electrophoresis of G-6-PD from sonicates of cultured skin cells. Samples were run in duplicate, starting from the origin which appears at the top of the figure. From left to right are shown the AB phenotype of 2 Negro females (double bands) and 3 individuals with single bands, 2 A's (fast band) and 1 B (slow band).

FIG. 2.—Electrophoretic pattern of G-6-PD from sonicates of cultured cells. Samples were run singly, starting from the origin at the top of the figure. From left to right are the AB phenotype of the cell culture from Mrs. De. prior to cloning, and the single bands of nine clones derived from the original cell lines. Variation in intensity of staining is due to inequality of enzyme concentration applied to the starch gel.

the inactivation of one, or at least part of one, X chromosome is most probably the mechanism of dosage compensation.

However, these findings do not imply that an entire X chromosome is inactivated in the female. Recent experiments involving the sex-linked Xg^a blood group have failed to show mosaicism.[16]

The probable mechanism of inactivation of an X chromosome is its transformation into heterochromatin. This belief is based on the observation that in the female one X chromosome is allocyclic,[17] replicates late,[18, 19] and probably forms the heterochromatic sex chromatin body.[20] However, inactivation of chromosomes by conversion to heterochromatin is not an all or none phenomenon either. In Drosophila, several genes are known to function even though located in the midst of heterochromatin,[21] and in a recent paper, Russell[22] has described experiments using translocations of marked autosomal fragments to the X chromosomes of female mice. It was shown that heterochromatin does not inactivate the entire attached fragment. Instead, there is a gradient of inactivation spreading only over limited distances.

Summary.—The appearance of two distinct populations of cells in the female heterozygous for both quantitative and qualitative G-6-PD variants is direct evidence in favor of the "Lyon Hypothesis." As far as the locus for G-6-PD is concerned, in each single cell only one X chromosome is functional. However, these data do not imply that one entire X chromosome is inactivated.

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† Trainee under Genetics Training Grant 2G–795 of the National Institutes of Health.

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*CHICK CEPHALOGENESIS, I. THE EFFECT OF RNA ON EARLY CEPHALIC DEVELOPMENT**

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Earlier studies[1] have shown that the short head process (cephalic notochord) and prechordal plate mesoderm do not reform when they are totally excised from cultured chick embryos which are in Hamburger-Hamilton stage 5 of development.[2] As a result, the forebrain, the midbrain, and their derivatives develop abnormally. These abnormalities include microcephaly, eye defects, acleiencephaly, and a twisting of the neural tube in the operated region. The remainder of the embryo, the hindbrain, trunk, and tail, develops normally. The reimplantation of the excised notochord to the operated embryo restores the normal development of brain.[3] It appears therefore that the mechanics of the phenomenon in question are similar to the classical primary induction demonstrated in the amphibians. In the latter, it was shown that the full differentiation of the neural rudiments could not take place in the absence of underlying chordamesoderm.[4, 5] Recent experiments have shown that this type of information transfer can be attributed to an "inductor substance" which has been shown to be of the nature of RNA.[6–8]

Experiments were designed therefore to test the hypothesis that RNA isolated from pooled notochords (N-RNA) could replace the extirpated notochord and cause the operated embryos to form fully differentiated fore- and midbrains. Head processes were removed from chick embryos at Hamburger-Hamilton stage 5. N-RNA was immediately applied to these operated embryos, and they were allowed to develop in culture for an additional 24 hr or until they had reached stage 9 or 10 of development. N-RNA was also added to unoperated embryos. In addition, brain RNA, chick liver RNA, whole chick RNA, whole chick minus brain and notochord RNA, or DNA extracted from calf liver was applied to operated and unoperated series of embryos. The aim of the communication is to report in detail our experimental data pertaining to the effect of specific RNA's in chick cephalogenesis, and to give preliminary data indicating the more generalized effect of DNA.

Experimental.—Materials: Fertilized eggs from white Leghorn hens were used within five days after laying. They were obtained from the Shaw Hatcheries in West Chester, Pennsylvania, and were stored at a temperature of 16–18° C. For uniform results, data were collected from only those experiments performed during the autumn, winter, and spring.[9]

Culture technique: The eggs were incubated approximately 19–22 hr at 38°C in a forced draft incubator to ensure the designated Hamburger-Hamilton stage 5 of development. The blastoderm was cultured according to the technique described by New.[10] By using this technique, the blastoderm can be cultured with the ventral surface uppermost, thus allowing the removal of the entoderm and notochord, without cutting the ectoderm. Microsurgery was done with tungsten needles. Following the operation, the embryos were treated separately with the various RNA's or with DNA. The treatment simply involves applying by micropipette 0.1–0.3 ml of 0.9% saline, or saline containing 0.5 mg RNA or 0.05 mg, 0.1 mg, or 0.2 mg of DNA to the cephalic region of the embryos.

Extraction of RNA: The RNA's were isolated from notochord (N-RNA), brain (B-RNA), chick liver (CL-RNA), whole chick (WC-RNA), and whole chick minus brain and notochord

(C-less-RNA) by a modified Kirby procedure.[6] The respective tissues were collected from approximately 85 embryos, 11–13 days. The pooled tissue was homogenized in the cold (2°C) with 6–10 volumes of saline (0.9% NaCl). An equal volume of water-saturated phenol was added to the homogenate, stirred with the Lourdes mixer (60 volts for 5–10 min), and then centrifuged 30 min at 2,000 rpm. The top aqueous layer was removed by suction, and to this was added potassium acetate up to 2% concentration followed by 2–3 volumes of 95% alcohol. The sediment was collected by centrifugation and redissolved in saline. Undissolved particles were removed by high-speed centrifugation. The RNA solution was washed with ether to remove contaminating phenol, and the ether was expelled under negative pressure; 2–3 volumes of ethanol were added to this solution and so kept in the deep freeze until used.

UV spectrophotometric examination of the isolated RNA gave a typical curve of nucleic acid with maximum and minimum absorption at 258 and 230 mμ, respectively. The ratio between 260/230 exceeded 2, and 280/260 was between 0.45 and 0.5. The color developed by treatment with orcinol reagent was green and with diphenylamine, blue. Routine tests for proteins were negative.

Extraction of DNA: Calf liver was minced with scissors. Eight volumes of 0.25 *M* sucrose containing 0.0033 *M* $CaCl_2$ and one volume of 0.5 *M* sucrose were added to the minced tissue. This sucrose containing the minced liver was then placed in a Waring blendor and mixed for 3 min at low speed and then 1 min at high speed. The mixture was filtered through doubled cheesecloth three times, and the filtrate centrifuged at 2,000 rpm for 10 min. The precipitate was collected and dispersed in two volumes of 0.25 sucrose containing 0.0033 *M* $CaCl_2$. Again it was filtered, spun at 2,000 rpm for 10 min, and the precipitate collected. This was repeated at least three times. The final precipitate was suspended in 1 *M* NaCl and stirred by magnetic stirrer for 16 hr at 2°C. It was then centrifuged at 30,000 rpm for 30 min, and the supernatant collected. To the supernatant was added six volumes of ice-cold distilled water, and the DNA fibers were removed. These fibers were collected by using a glass rod and were then put into 6% para-aminosalicylic acid in 1 *M* NaCl and stirred for 10 min with a Magnastir. An equal volume of 90% water-saturated phenol was next added, and the whole preparation stirred for 30 min at 2°C. The mixture was then centrifuged at 2,000 rpm for 30 min. The aqueous layer was removed, and the DNA precipitated with two volumes of ethanol. The fibers were again removed with a glass rod and resuspended in 0.9% NaCl by shaker. This technique for the isolation of DNA was devised by Philip Nash of this laboratory.

The UV spectrophotometric examination of the isolated DNA gave a typical curve of nucleic acid with a maximum absorption at 258 mμ and a minimum absorption at 230 mμ. The ratio between 260/230 exceeded 2, and the 280/260 ratio was between 0.45 and 0.5. Routine purity tests indicated that the samples contained 28 μg RNA/ml (9.5%), 240 μg DNA/ml (81.7), and 26 μg protein/ml (8.8%).

Results.—Effects of RNA on operated embryos: N-RNA: The short head processes were removed from 60 embryos. Forty of these embryos were treated with N-RNA, and twenty received no treatment. These latter embryos had midbrain and forebrain abnormalities and optic defects. Sections showed that the excised portion of the notochord had not reformed.

Of the 40 embryos which were operated on and treated with N-RNA, 31 appeared normal in their development; one had two forebrains and midbrains (Fig. 1), and the remaining 8 developed with the expected abnormalities, i.e., microcephaly, acleiencephaly, and twisting of the neural tube. Sections of these 8 revealed the absence of notochords, whereas, in the 31 which developed normally, varying amounts of the notochord had reformed in the operated regions. In 4 of these latter cases (Fig. 2), two cephalic notochords had formed. The newly formed notochords were not always found midventral to the brain. Often they were ventrolateral to the neural tubes. As yet, no studies have been undertaken to determine the source of cells from which the new notochords were derived.

B-RNA: The short head processes were extirpated from 45 cultured chick

embryos. B-RNA was added to the excised region of all blastoderms. Twenty additional embryos were operated on but not treated, and all of them showed varying degrees of cephalic abnormalities in the fore- and midbrain regions. In none of the latter series did the notochords reform.

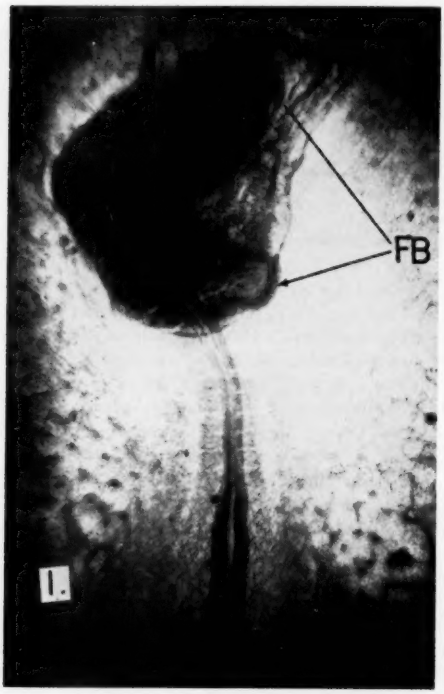

FIG. 1.—Short head process removed from stage 5 embryo and embryo treated with 0.5 mg N-RNA. Note duplication of brain. ×20. FB, Forebrain.

Of the 45 embryos treated with B-RNA, 23 had normal brain development, and 3 embryos had developed 2 complete brains (Fig. 3). In none of these embryos did the notochord reform in the regions from which it had been extirpated.

Effects of RNA on unoperated cultured chick embryos: N-RNA: For this series of experiments 30 embryos were cultured, and N-RNA was added to the presumptive fore- and midbrain regions of the blastoderm. An equal number were used as controls, and these developed normally. The treated embryos also developed normally. Gross examination indicated, however, an enlargement of the notochord in the trunk region of 6 embryos. Five of the remaining 24 embryos showed an increase in size of the notochord in the cephalic region. Normally, a cross section of the cephalic notochords of embryos of Hamburger-Hamilton stages 9 and 10 contains approximately 10 cells (Fig. 4). The number of cells in the experimental embryos was tripled or quadrupled at various levels along the length of the cephalic notochord (Fig. 5). No other tissue or structure was found to be affected by the N-RNA treatment.

B-RNA: B-RNA was added to 35 intact stage 5 embryos. Gross examination indicated normal development. Sectional study of these embryos revealed, however, the presence of thickened ectoderm dorsal to the brain, suggesting the development of a secondary medullary plate (Fig. 6). This thickened ectoderm was found in 7 of the 35 embryos. Another commonly encountered result of this treatment was a proliferation of loose, unorganized aggregates of brain cells into the neurocoele (Fig. 7). It was noted that in some embryos the neurocoele was almost obliterated by these cells. No other structure was affected by the B-RNA.

The effect of CL-RNA, WC-RNA and C-less-RNA, on operated and unoperated embryos: The short head processes were removed from 120 embryos. To 35 of these was added CL-RNA; to 30, WC-RNA; and to 55, C-less-RNA. The forebrains and midbrains did not develop normally in any of these embryos, and in

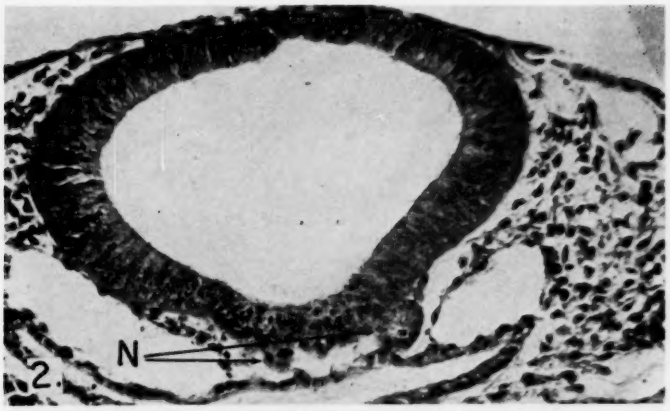

FIG. 2.—X-S of embryo from which the short head process was removed and 0.5 mg N-RNA added. Note formation of two notochords. × 65. N, Notochord.

none were there found reformed notochords, excessive brain cells, or secondary medullary plates. Equal numbers of intact embryos were treated with the RNA's, and all developed normally. Studies of serial sections of these embryos showed the normal development of all the embryonic structures.

The effect of DNA on operated and unoperated embryos: Calf liver DNA (0.2 mg) was applied to 40 intact cultured stage 5 embryos which were allowed to develop for an additional 24 hr. This concentration of DNA had an adverse effect on 29 of the treated embryos. These embryos showed defects such as stunted development, cardia bifida, abnormal body folds, and lack of fusion of the neural folds. All regions of the 29 embryos appeared to be adversely affected by this treatment. The remaining 11 embryos ap-

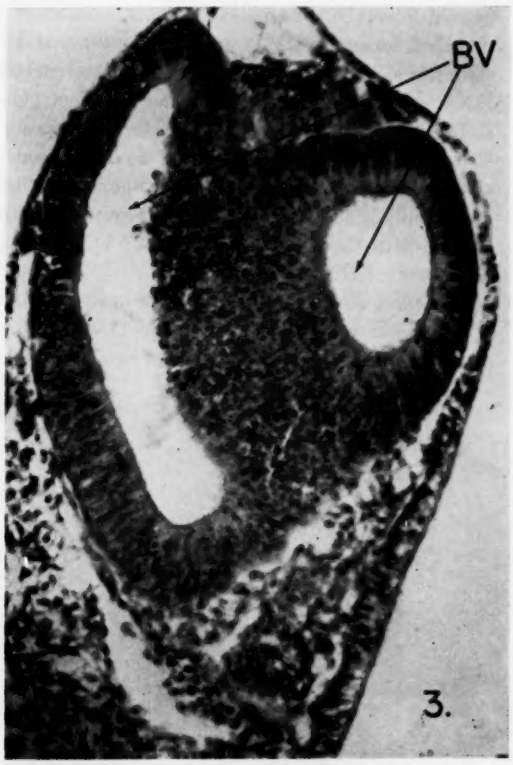

FIG. 3.—X-S of embryo from which head process was removed and 0.5 mg B-RNA added. Note double brain vesicles and absence of notochord. × 65. BV, Brain Vesicle.

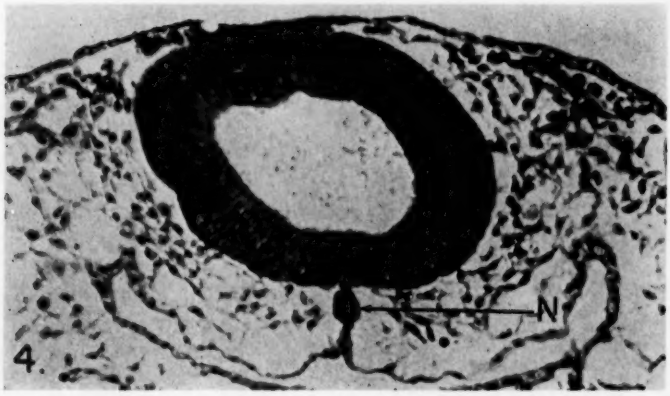

FIG. 4.—X-S of hindbrain of a control stage 10 embryo showing normal size of notochord. × 65. N, Notochord.

peared normal in their development. A study of serial sections of these embryos revealed, however, the increase in size of entodermal, mesodermal, and ectodermal structures, as well as duplications of structures. In no case was the effect of DNA specific but appeared rather to affect generally the entire embryo.

The lesser concentrations of DNA (0.05 mg and 0.1 mg) were used to treat 80 embryos. These amounts caused fewer defects. Only 7 of the total of 80 showed gross abnormalities which included stunting, cardia bifida, acleiencephaly, and acephaly. A study of sections of these embryos revealed fewer histological effects from these concentrations of DNA than was found with the greater concentration of DNA.

The effect of DNA on operated embryos: Because of the lesser teratogenic effect of the lower concentrations of the DNA, 0.05 mg was added to 50 embryos and 0.1 mg to 50 embryos. The short head processes had been removed from all these

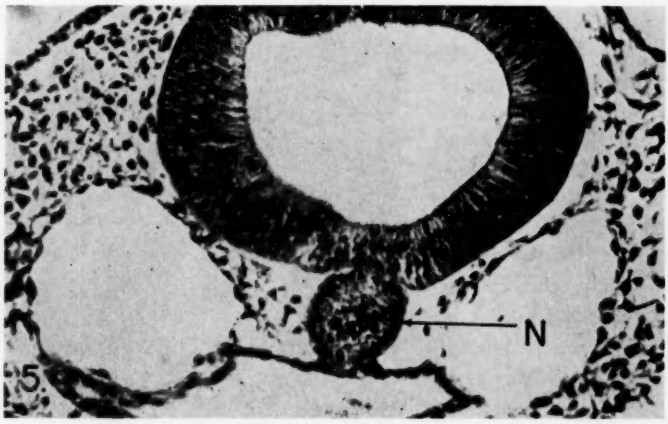

FIG. 5.—X-S of hindbrain of experimental stage 10 embryo showing increased size of notochord. Intact chick treated with 0.5 mg N-RNA. × 65. N, Notochord.

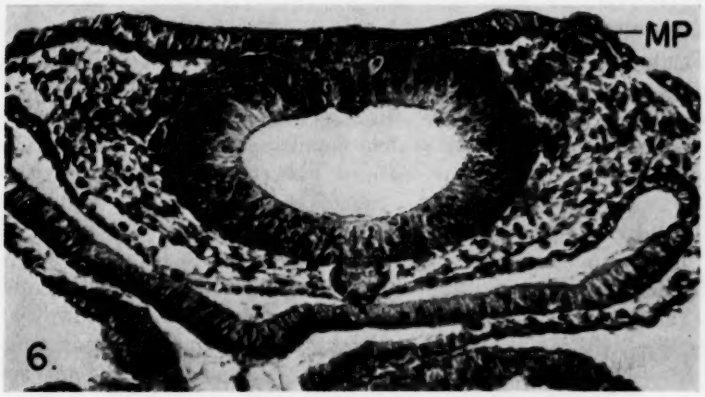

FIG. 6.—B-RNA added to intact stage 5 embryo. Note thickened ectoderm dorsal to neural tube. × 65. MP, Medullary Plate.

embryos. In no case was there a reformation of the excised notochord, and in all cases the embryos developed with abnormal fore- and midbrains. Detailed information of the effect of DNA on the developing embryos will be presented in a later publication.

Discussion.—The effect of an exogenous substance on an undifferentiated cell system causing differentiation of that system is a phenomenon termed induction. Differentiation itself can be expressed in terms of the formation of new structures, new proteins (enzymes) or both. The classical experiment of Mangold[4] demonstrates the capacity of the young gastrula ectoderm to develop into components of the axial structures when associated with the underlying chordamesoderm. In this type of induction, primary induction, there is no structural or functional similarity between the ectodermal and chordamesodermal derivatives.

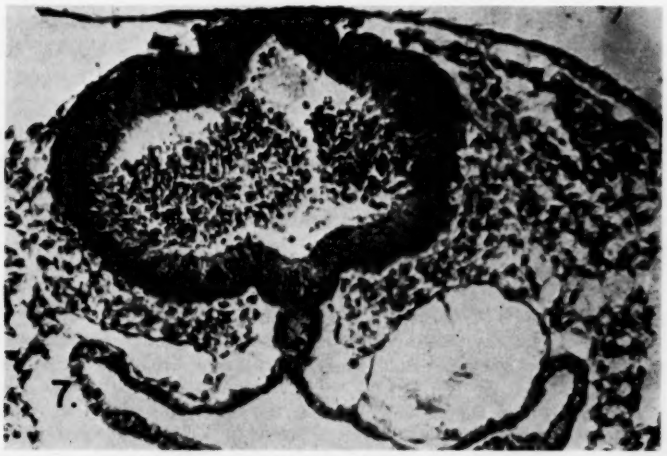

FIG. 7.—B-RNA added to intact stage 5 embryo. Note proliferation of brain cells into the neurocoele. × 65.

It has also been shown that chordamesoderm discharges active substances into its surrounding medium.[11] These diffusible substances were found to be ribonucleoprotein (RP). They are responsible for the differentiation of explanted gastrula ectoderm.[12] In this case, RP refers to a mixture of microsomes and artificial aggregates of RNA and protein appearing during the process of experimentation. In this sense, it is interesting to note that most workers in the field of embryonic induction agree that RP is capable of initiating ectodermal differentiation. Present experiments being conducted in this laboratory,[13] however, indicate that RP or microsomes isolated from pooled brains of 11–13 day chick embryos and applied to stage 5 blastoderms from which the head processes are removed have no effect on their embryogenesis, and they develop as their operated controls. If, on the other hand, B-RNA is applied to these operated embryos, normal brains develop. No other structures are affected. Furthermore, B-RNA can cause the intact stage 5 embryos to form an excessive growth of nerve cells or can initiate the growth of a second brain. These findings illustrate clearly the specific function of B-RNA.

The specificity of notochordal RNA was shown by its capacity to cause the enlargement or duplication of the host's notochord. When this RNA was used to treat stage 5 embryos without head processes, notochord reappeared in the region of extirpation and the fore- and midbrains developed more normally.

Results have been reported to show that a factor responsible for specific differentiation is RNA.[6, 14] Once the active RNA enters the cells, the responsive cells begin to synthesize specific protein or enzyme, thus leading to structural and functional cell types characteristic of the RNA tissue source.

This type of induction can be correlated with the work of Mangold and Spemann.[15] These workers found that the pregastrular ectoderm would not induce differentiation, whereas the postgastrular medullary plate demonstrated this capacity. The medullary plate when transplanted into the blastocoele of young gastrulae induced a secondary medullary plate in the host. This phenomenon, termed "homoiogenetic induction," is similar to the specific inductive capacity demonstrated by RNA. The observations of the effects of DNA on the chick embryo have thus far indicated that the latter does not have a specific effect on the blastoderm.

Summary.—Stage 5 chick embryos were explanted *in vitro*. They were employed to test for the function of RNA isolated from the pooled brains or notochords of 11–13 day chick embryos. It was found that brain and notochord RNA can initiate, respectively, the development of brain and notochord. Therefore, brain and notochord RNA's carry information which will initiate the differentiation of their respective tissues.

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† Postdoctoral fellow, National Institutes of Health, BPD-16, 189.

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HYDROCORTISONE-STIMULATED SYNTHESIS OF NUCLEAR RNA IN ENZYME INDUCTION

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Earlier reports from this laboratory have demonstrated that the hydrocortisone-mediated induction of tyrosine transaminase in rat liver involves an increased rate of enzyme synthesis.[1, 2] Other evidence[3, 4] is consistent with the conclusion that enzyme synthesis is required in similar inductions of other hepatic enzymes, and Segal and Hopper[5] recently described a direct demonstration of increased synthesis of the glutamic-alanine transaminase. There is thus ample evidence to support the hypothesis that the basis for adrenocorticoid effects on hepatic metabolism lies in enzyme induction, and the mechanism of hormonal action in these inductions thereby assumes considerable significance. Experience with microbial systems has shown that induced synthesis of enzyme proteins reflects a primary alteration in the rate of formation of a specific fraction of RNA. In a study of corticosteroid effects on RNA turnover in rat liver, Feigelson, Gross, and Feigelson[6] found that precursor incorporation into RNA was increased in all the subcellular fractions of liver. This question has been reinvestigated using pulse-labeling techniques, and the results of these investigations are reported here, together with studies on the characterization of the RNA formed in response to hormone administration. A preliminary report describing somewhat similar studies was recently presented by Garren and Howell.[7]

Materials and Methods.—Male Sprague-Dawley rats weighing 150–200 gm were adrenalectomized 24–48 hr, and fasted for 18–24 hr before experiments. Induced animals received 5 mg of hydrocortisone intraperitoneally. Carrier-free $Na_2HP^{32}O_4$ (1 mc) was also given intraperitoneally, and in pulse-labeling experiments was given 20 min before the animals were killed. Livers were quick-frozen and stored in liquid nitrogen for some experiments, or placed in cold 0.25 *M* sucrose–0.001 *M* $MgCl_2$ (SM) and fractionated immediately in others.

Preparation of liver fractions: Livers were homogenized in 4 volumes of SM, and the homogenates filtered twice through several layers of cheesecloth. The nuclear fraction was isolated by centrifugation at 600 × *g* for 5 min, resuspended in a volume of SM equal to the liver weight, and recentrifuged. This wash was added to the supernatant of the first centrifugation to constitute the cytoplasmic fraction. The nuclear fraction was washed a second time with 10 volumes of SM, then suspended in 0.01 *M* Tris, pH 7.6, 0.001 *M* $MgCl_2$, in a volume equal to twice the liver weight. Temperature was maintained at 0–3°C during all preparative steps.

Isolation of RNA: Early experiments showed that when RNA was isolated by either of the phenol methods described below, or by the method of Davidson and Smellie,[8] both variation in the extent of extraction of labeled RNA and inclusion of radioactive contaminants were so great that specific radioactivity measurements were not reproducible. Hence, when specific radioactivity was the parameter measured, RNA was isolated by the following hot salt-DEAE procedure.

Aliquots of each fraction containing 600–900 μg RNA were extracted three times in 2.0 *M* NaCl–0.015 *M* phosphate, pH 7, at 100°. Extracts were pooled, 2 volumes of cold ethanol added, and the nucleic acids were collected after $1^1/_2$ hr precipitation at 4°. The yield of RNA at this point, calculated from average values,[9] ranged from 80 to 108% and averaged over 90%. Removal of radioactive contaminants from these preparations was based on the observations of Jacobson and Nishimura[10] concerning the behavior of RNA on DEAE paper. Aliquots containing 200–500 μg RNA were spotted on 15 × 7-cm strips of DEAE paper (chloride form, Whatman) cut to form a point at one edge. These were then irrigated with 0.4 *M* NaCl for 12 hr, then briefly with 70% ethanol to remove salt, and finally with ether:ethanol:chloroform (2:2:1), again for 12 hr. RNA was then eluted from the paper with 0.5 *N* KOH; ultraviolet absorbancy was completely removed after 3–4 ml were collected. The KOH eluate was incubated at 37° for 18 hr, and the hydrolysate was then acidified to 0.5 *N* $HClO_4$ to remove DNA and any residual protein. The clear solutions were analyzed for RNA by ultraviolet measurements and for P^{32} by liquid scintillation counting. Virtually all of the RNA added to the paper was recovered as nucleotides. Acid-washed Norit removed 95–100% of the radioactivity, indicating that labeled contaminants were negligible. The mononucleotides were eluted from the charcoal and chromatographed on DEAE paper as described by Jacobson;[11] no radioactive areas were detected except those corresponding to the carrier nucleotides added prior to chromatography. These areas were cut out and counted directly by liquid scintillation counting to estimate the base ratios of the newly synthesized RNA. RNA-specific radioactivity measurements were corrected for variation in the specific radioactivity of the acid-soluble phosphate of each homogenate. In some experiments, radioactivity of the phosphate adsorbed by charcoal and released in 1 *N* HCl at 100° for 10 min was determined. Specific radioactivity of this phosphate was close to that of the inorganic phosphate, and variation in one was always paralleled by the other, and hence the measurements of total acid-soluble phosphate were considered sufficient.

For studies of size distribution and biological activity, nuclear RNA was usually prepared by a method (phenol extraction) similar to that described by Hiatt,[12] employing dodecyl sulfate. In the experiments described, no attempt was made to remove the products of limited (3–5 min) DNAase digestion, and DNA (measured by diphenylamine[13]) was present in amounts roughly equivalent to RNA. The specific radioactivity of the product was usually, but not always, approximately that expected from the results of the hot salt-DEAE procedure. Radioactivity of DNA was assumed to be negligible. For some experiments, deoxycholate was employed in place of dodecyl sulfate. Under these conditions little or no DNA was present in the product, but only 50–60% of the radioactive RNA was extracted. Cytoplasmic RNA was prepared by phenol-dodecyl sulfate extraction, DNAase treatment and ancillary steps being omitted. All the RNA preparations obtained by phenol extraction had a 260/280 ratio greater than 1.9.

Analytical procedures: Sucrose gradients were prepared and sampled as described by Britten and Roberts.[14] Aliquots containing 0.6–1 mg RNA were layered over 4.7 ml of a linear gradient 3–20% sucrose, containing 0.01 *M* Tris, pH 7.6, and 0.001 *M* $MgCl_2$. Centrifugation was carried out for 3.75 hr at 4° at 39,000 rpm. Aliquots of each 2-drop fraction were pipetted onto filter paper disks and then washed and counted as described by Bollum.[15] The portion of each fraction remaining was diluted to 2.5 ml for absorbancy determinations at 260 mμ. Cytoplasmic fractions were assayed for tyrosine-α-ketoglutarate transaminase as described previously.[16] Ribosomes and soluble fractions used in amino acid incorporation studies were prepared from the livers of fasted, intact rats by modifications of the procedure of Robinson and Novelli.[17] The incorporation assay mixture contained, in 1.0 ml: 50 μmoles Tris, pH 7.6; 9 μmoles $MgCl_2$; 50 μmoles KCl; 20 μmoles β-mercaptoethanol; 1 μmole ATP; 0.5 μmole GTP; 10 μmoles phosphopyruvate; 50 μg pyruvic kinase; 0.5 μc C^{14}-leucine, 25 μc per μmole; 30 μg of each of 19 amino acids (leucine omitted); 200 μg sRNA, 10 μg DNA-ase, 1.0 mg ribosomal protein, and 2.75 mg soluble protein. Rat liver sRNA was prepared as described by Brunngraber,[18] except that the product was treated with

DNAase and rechromatographed on DEAE. When tested under conditions used for incorporation assay (ribosomes omitted), this sRNA accepted 2 mμmoles C^{14}-leucine per mg, dilution of isotope by endogenous leucine being ignored. Amino acid incorporation was assayed by the method of Mans and Novelli.[19]

Results.—Rate of RNA synthesis in nuclear and cytoplasmic fractions: Figure 1 demonstrates the effect of hydrocortisone on the rate of RNA synthesis, as measured by pulse labeling, during enzyme induction. The rate of synthesis of cytoplasmic RNA is unchanged by the hormone treatment. Nuclear RNA synthesis in noninduced (0 time) animals is 3–4 times faster than that of cytoplasmic RNA, as has been repeatedly observed by others. Hydrocortisone administration increases the rate of synthesis of nuclear RNA, the effect becoming marked after about 60 min, and increasing to about 3-fold by 4 hr. The rapid rise in the rate of nuclear RNA synthesis after 60 min is accompanied by a gradual accumulation of the induced transaminase. After 2 hr, while the rate of nuclear RNA synthesis increases more gradually, enzyme begins to accumulate rapidly. These results are consistent with the concept that enzyme induction is the secondary result of a primary hormonal effect on the synthesis of nuclear RNA. The delayed response in enzyme level is not unexpected in that an increase in this parameter requires enzyme accumulation.

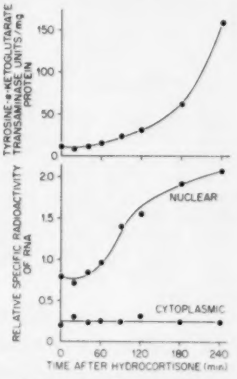

FIG. 1.—Rates of synthesis of nuclear and cytoplasmic RNA during enzyme induction. Each point represents a determination on a single animal; hydrocortisone was given to all at 0 time, and P^{32} to each animal 20 min before it was killed. Relative specific radioactivity is cpm per mg RNA/cpm per μmole acid-soluble Pi × 100. RNA was prepared and analyzed by the hot salt-DEAE method.

Since the rapidity with which hydrocortisone stimulates RNA synthesis is of importance in consideration of the detailed mechanisms of hormone action, the earlier effects of the hormone were examined more closely. Figure 2 presents the data of such analyses, and also demonstrates the reproducibility of these measurements. Hydrocortisone administration appears to cause a slight (but reproducible, cf. Fig. 1) immediate depression in nuclear RNA synthesis. Increased synthesis of nuclear RNA begins after a delay of about 30 min. Enzyme activity is increased over the control level after 60 min. Since, as stated above, this requires an accumulation of newly synthesized enzyme, these data suggest that nuclear RNA synthesized in response to hydrocortisone is quite rapidly utilized in enzyme synthesis.

When P^{32} is present for 2 hr or longer during hormone treatment, an effect on labeling of cytoplasmic as well as nuclear RNA is seen (Fig. 3), a result in agreement with that of Feigelson *et al.*[6] From the data of Figures 1 and 2, it is clear that hormonally increased labeling of cytoplasmic RNA reflects RNA synthesized in the nucleus. These results can be interpreted as indicating that the nuclear RNA detected by pulse-labeling passes, in time, into the cytoplasm. That the induced transaminase is synthesized on cytoplasmic ribosomes was suggested by the detection of an enzymically inactive, immunologically crossreactive material bound to this fraction.[1]

RNA characterization: (1) *Sucrose gradient analysis:* The sedimentation of

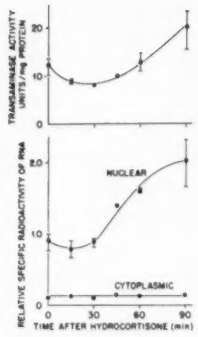

FIG. 2.—Early time-course of the hydrocortisone effect on enzyme and RNA synthesis. Vertical bars indicate the range of values from determinations on 2 or 3 animals. Other details as in Fig. 1.

rapidly labeled nuclear RNA from induced and noninduced liver is seen in Figure 4. The products of limited DNAase digestion appear as a large peak overlying the 5 S (Svedberg units) component of nuclear RNA, which normally appears as a small peak in fractions 25–27 under these conditions. Experiments wherein the partially degraded DNA was removed, as described by Hiatt,[12] established that sedimentation of both bulk and labeled RNA was unchanged by the presence of DNA. As shown by Hiatt, the pulse-labeled nuclear RNA is heterogeneous, ranging in size from about 7–40 S. The pulse-labeled RNA from induced livers sediments much like that prepared from noninduced livers; the apparent shift toward higher molecular weight cannot be considered significant in view of the extensive variation that we have observed in many similar analyses. No indication of an increase in a specific fraction of the rapidly labeled RNA was found. Similarly, when RNA was prepared using deoxycholate there was no change due to induction (Fig. 5).

In sucrose gradient experiments livers were cooled in an ice bath, rather than quick-frozen in liquid nitrogen as was done in the previous analytical experiments. The slower cooling rate appears to permit passage of some of the pulse-labeled nuclear RNA into the cytoplasm, as is indicated by the analysis of cytoplasmic RNA prepared from these livers (Fig. 6). Sedimentation of the RNA from control livers yields a labeling pattern similar to that observed by Hiatt,[12] with nearly all the radioactivity being associated with the slowest absorbancy peak, presumably containing transfer RNA. In the preparations analyzed here, the specific radioactivity of the cytoplasmic RNA from induced livers was nearly twice that from control livers, and the induced nuclear RNA was correspondingly lower in radioactivity than expected. The excess-labeled cytoplasmic RNA, known to be of nuclear origin since its formation is stimulated by induction, can be seen to sediment in a broad peak beginning at about fraction 16 (*ca.* 20 S), then increasing in amount and forming a shoulder (fraction

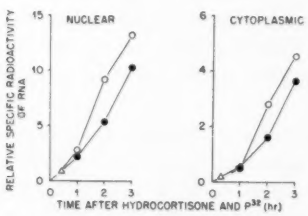

FIG. 3.—Hydrocortisone effects on long-term P^{32}-labeling of RNA. Isotope was given to all animals at 0 time; induced animals (open circles) received hydrocortisone simultaneously. The triangles represent points from pulse-labeling experiments. Other details as in Fig. 1.

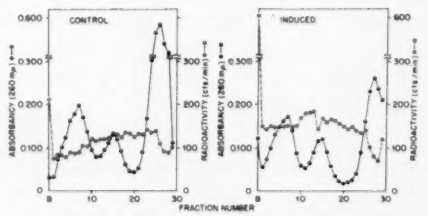

FIG. 4.—Sucrose gradient analysis of pulse-labeled nuclear RNA. Livers from 6 similarly treated animals were pooled for each group. Induced animals were given hydrocortisone 2 hr, and P^{32} 20 min, before death; controls received only P^{32}. RNA was prepared by the phenol-dodecyl sulfate method. Specific radioactivity of the induced preparation was 1.57 times that from controls.

21) on, and merging with, the peak of labeled transfer RNA. This sedimentation pattern is similar to that of the cytoplasmic RNA recently described by Hoagland and Askonas,[20] which these authors found to have certain properties in common with bacterial messenger RNA. The discrepancy in size between the rapidly labeled RNA of the nucleus (7 to >40 S) and the same RNA when found in the cytoplasm (<20 S) may reflect some degradation of this RNA by nucleases.

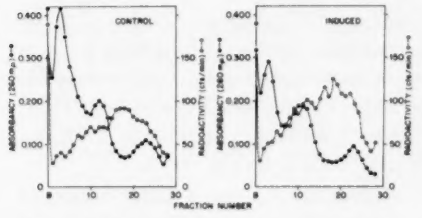

FIG. 5.—Sucrose gradient analysis of pulse-labeled nuclear RNA prepared without dodecyl sulfate. Centrifugation was at 10°. Specific radioactivities of induced/control = 1.78. Other details as in Fig. 4.

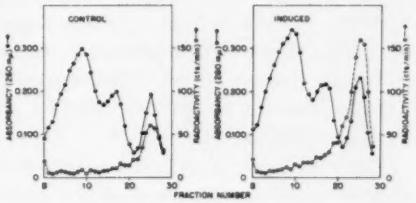

FIG. 6.—Sucrose gradient analysis of pulse-labeled cytoplasmic RNA. Specific radioactivities of induced/control = 1.87. Other details as in Fig. 4.

(2) *Activity in amino acid incorporation:* Nuclear RNA prepared by the phenol-dodecyl sulfate method was found to have little or no effect on amino acid incorporation in the rat liver system described. Hoagland and Askonas have suggested[20] that such inactivity may be due to this detergent, and accordingly we have employed nuclear RNA prepared using deoxycholate in place of dodecyl sulfate. These RNA preparations were somewhat active in stimulating leucine incorporation into protein, and response was approximately linear with amounts up to 1 mg (Fig. 7). RNA from induced livers consistently was slightly more active (5–10 per cent) in these assays, but never to the extent of the difference in specific radioactivity due to induction (2- to 3-fold). When assayed for charging capacity, nuclear RNA preparations were found to stimulate leucine incorporation into RNA with an efficiency about 10 per cent of that of highly purified sRNA, and there was no change due to induction. Assays of leucine incorporation into protein were done with sRNA added at twice the saturating level, and hence it is felt that stimulation by nuclear RNA could not be due to addition of transfer RNA. That the system employed is responsive to addition of messenger RNA was demonstrated in experiments wherein C^{14}-phenylalanine incorporation was doubled by addition of 40 μg polyuridylic acid (Miles Laboratories), while C^{14}-leucine incorporation was unchanged.

(3) *Base composition:* The base ratios of the pulse-labeled RNA, as determined by isotopic labeling, are indicated in Table 1. The data presented are average values from 2 groups of rats, 1 untreated and the other given hydrocortisone for 1 hr. Other groups were analyzed over a 4-hr period of hormone treatment, and in no case was there a marked change from the values in Table 1. The composition of the rapidly labeled nuclear RNA is

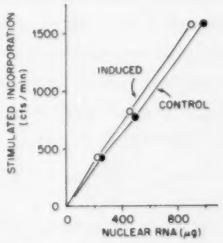

FIG. 7.—Stimulation of C^{14}-leucine incorporation into protein by nuclear RNA. Isotope incorporation by the system without added nuclear RNA was 11,060 cpm in a 60 min incubation.

TABLE 1

BASE COMPOSITION OF PULSE-LABELED RNA*

Fraction	Per Cent of Total Radioactivity in— Cytidylic	Guanylic	Adenylic	Uridylic	Ratio (A + U/C + G)
Nuclear	26.5	27.0	21.1	25.2	0.87
Cytoplasmic	22.6	23.2	29.0	24.9	1.18

* RNA prepared and analyzed by the hot salt-DEAE method. The numbers are the averages of duplicate determinations on 2 groups of 3 rats given P^{32} for 20 min. Variation between groups was about ±2%.

similar to that of the total nuclear RNA, determined chemically (ratio of A + U/C + G = 0.81; ref. 21). Composition of the pulse-labeled cytoplasmic RNA is not consistent with labeling owing to turnover of the terminal cytidylic and adenylic residues of transfer RNA. The composition is also unlike that of the nuclear RNA, analyzed from the same livers, nor is it like either microsomal or cell sap RNA determined chemically (A + U/C + G = 0.60 and 0.63, respectively[21]).

Discussion.—The functional nature of the nuclear RNA formed under hormonal influence is difficult to assess at this time. That an increased synthesis of nuclear RNA precedes increased enzyme synthesis suggests, in the light of current theories of enzyme induction,[22] that the hormone stimulates formation of messenger RNA. The rapidly labeled RNA of the nucleus shares some characteristics with microbial messenger (cf. Hiatt[12]), and hydrocortisone stimulates the synthesis of this fraction. However, that the induced RNA could be wholly or even largely functional as messenger is not entirely in accord with our present knowledge of hydrocortisone effects on hepatic protein synthesis. Isotope incorporation into soluble liver proteins *in vivo* is increased 10–20 per cent in the first hours of hormone treatment,[2] a result consistent with the selective induction of a few enzymes, but not proportional to the large increase in nuclear RNA synthesis observed here. These considerations point to the possibility that only a small fraction of the induced RNA is active as messenger, as suggested by our results in assays for messenger activity *in vitro*.

Summary.—Hydrocortisone administration to adrenalectomized rats results in a 2- to 3-fold stimulation of the rate of liver nuclear RNA synthesis, as measured by pulse-labeling techniques. Labeling of cytoplasmic RNA is unchanged in pulse experiments, but is increased by the hormone if isotope is given for longer periods of time. Stimulation of nuclear RNA synthesis begins after a lag of about 30 min duration, and accumulation of the induced tyrosine transaminase begins about 30 min after the effect on RNA is apparent. The sedimentation distribution and base composition of the induced RNA are indistinguishable from those of RNA that is pulse-labeled in the absence of hormone. Nuclear RNA from induced livers was slightly more active than that from noninduced controls in stimulating amino acid incorporation *in vitro*.

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† Operated by Union Carbide Corporation for the U.S. Atomic Energy Commission.

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*STUDIES ON THE GRAM-NEGATIVE CELL WALL, I. EVIDENCE FOR THE ROLE OF 2-KETO-3-DEOXYOCTONATE IN THE LIPOPOLYSACCHARIDE OF SALMONELLA TYPHIMURIUM**

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The cell wall lipopolysaccharides which determine the somatic (o) antigen specificities of *Salmonella typhimurium* and *E. coli* consist of highly branched, complex polysaccharides linked to a glucosamine-containing lipid.[1] The polysaccharide moiety may contain as many as 6 neutral sugars; for example, L-glycero-D-mannoheptose, glucose, galactose, mannose, rhamnose, and abequose (3,6-dideoxy-D-galactose) have been identified[2, 3] as components of the polysaccharide of *S. typhimurium*. The polysaccharides are thought[3–5] to consist of an internal core structure, possibly similar in all enteric bacteria, to which are attached complex side chains bearing group or species specific antigenic determinants. New insight into both structure and mechanism of biosynthesis of these polysaccharides has recently been obtained[3, 5] through the use of mutant organisms deficient in the synthesis of specific nucleotide sugars. It was first shown by Nikaido[5, 6] that incomplete polysaccharides are formed by mutants which are unable to synthesize UDP-galactose as a result of loss of the enzyme, UDP-galactose-4-epimerase. The polysaccharides formed by these mutants characteristically lack not only galactose, but also certain other sugars present in the normal polymers, and appear to represent the innermost core region of the wild-type polysaccharide. Previous studies in our laboratory[3] on a UDP-galactose-4-epimeraseless strain of *S. typhimurium* have shown that the incomplete "core" polysaccharide formed by this mutant contains glucose, L-glycero-D-mannoheptose, and phosphate. It has now been found that this polysaccharide also contains 2-keto-3-deoxyoctonate (KDO),[7] recently identified by Heath and Ghalambor[8] as a component of the lipopolysaccharide of

E. coli 0111. In agreement with the results of these workers, the bulk of the KDO present in the lipopolysaccharide of the *S. typhimurium* mutant appears to be in glycosidic linkage at nonreducing terminal positions. In addition, however, approximately 25 per cent of the total KDO occurs in a different position; this fraction is recovered in the purified, lipid-free polysaccharide, and occupies the reducing-terminal position of glucose-heptose-phosphate chains. Most, if not all, of the polysaccharide chains appear to contain KDO at the reducing end, suggesting that the sugar acid may be involved in the linkage of the core polysaccharide to the lipid moiety of the intact lipopolysaccharide.

Materials and Methods.—The previously described[3] mutant of *S. typhimurium* lacking UDP-galactose-4-epimerase was grown commercially by the Grain Processing Corp. of Muscatine, Iowa, in a galactose-free peptone medium. Lipopolysaccharide was routinely prepared by phenol extraction of cell walls followed by precipitation with Mg^{++} as described earlier.[3] Free KDO was isolated by chromatography on DEAE cellulose as described in the legend for Figure 2 after hydrolysis of lipopolysaccharide at pH 3.4, and was purified either by adsorption and elution from charcoal according to Heath and Ghalambor,[8] or by paper chromatography on Whatman no. 40 paper. Solvent systems employed for identification of KDO were: 2-butanone, acetic acid, H_2O (8:1:1); ethyl acetate, acetic acid, H_2O (3:1:3); n-butanol, pyridine, 0.1 *N* HCl (5:3:2); ethanol, acetic acid, H_2O (80:1:19). 2-Keto-3-deoxyheptonolactone was a gift from Dr. D. B. Sprinson.

KDO was determined by the thiobarbituric acid method of Weissbach and Hurwitz[9] with minor modifications. For determination of KDO in the presence of a large excess of polysaccharide, the amounts of HIO_4 and $NaAsO_2$ were increased to 10 μmoles and 100 μmoles, respectively. A heating time of 20 min was required to give maximal color development with polysaccharide-bound KDO. Under the assay conditions, 1.0 μmole of KDO gave an absorbancy of 19.0 at 548 mμ in the Beckman DU spectrophotometer. Total lipopolysaccharide-bound KDO was determined after hydrolysis of lipopolysaccharide in 0.02 *N* H_2SO_4 for 20 min at 100°.

Heptose was determined by the cysteine-H_2SO_4 reaction,[10] modified as follows: 2.25 ml of H_2SO_4 (6 vol concentrated H_2SO_4 : 1 vol H_2O) were slowly added to duplicate samples (0.5 ml) in an ice-H_2O bath, and mixed by shaking in the cold. After 3 min, the tubes were transferred to a 20° bath for 3 min, and then heated in a vigorously boiling H_2O bath for exactly 10 min. The 10 min heating time minimized interference by other components of the polysaccharide. After cooling, 0.05 ml of 3% cysteine-HCl was added to one sample, the other serving as a blank. Absorbancy at 505 mμ and 545 mμ was determined exactly 2 hr after addition of cysteine, and corrected for nonspecific absorbance in the blank minus cysteine. Under these conditions, 1.0 μmole of L-glycero-D-mannoheptose gave a value of $A_{505} - A_{545} = 1.07$.

Glucose was determined with glucose oxidase (Worthington Glucostat reagents) after hydrolysis of the polysaccharide in 1 *N* HCl for 5 hr at 100° in sealed, evacuated tubes. Internal standards were included, and the values reported have been corrected for 10–15% loss of added glucose during hydrolysis.

Total carbohydrate was determined by the phenol-H_2SO_4 method,[11] reducing sugar by the Nelson method[12] and phosphate by the procedure of Berenblum and Chain[13] as modified by Ennor and Stocken.[14]

Results.—Identification of KDO in the lipopolysaccharide of the epimeraseless mutant: The presence of an unknown component was first suggested by the observation that the spectrum of the purified lipopolysaccharide in the cysteine-H_2SO_4 reaction contained a peak at 385–390 mμ which could not be assigned to any of the known constituents. The material responsible for this reaction could be released from lipopolysaccharide by mild acid hydrolysis and purified as described in *Materials and Methods.* The purified compound showed a distinctive absorption spectrum in the cysteine-H_2SO_4 reaction (λ_{max} = 383 mμ before cysteine, 390 mμ after cysteine addition, with diffuse absorption from 450–650 mμ, and a secondary peak at 590 mμ which appeared at 24–48 hr), and gave a reaction in the thiobar-

bituric acid test characteristic of 2-keto-3-deoxyaldonic acids (λ_{max} = 549 mμ). The presence of an α-keto acid was confirmed by formation of the semicarbazone derivative[15] (λ_{max} = 250 mμ). The isolated material was free of nitrogen and phosphorus, and was chromatographically identical in 4 solvent systems with authentic KDO, kindly supplied by Dr. E. C. Heath.[16] Identification of the *S. typhimurium* component as KDO was confirmed by periodate oxidation, which yielded HCHO, HCOOH, and formylpyruvate in the expected ratio of 1:3:1.

The present results support the conclusion of Heath and Ghalambor[8] that KDO exists in glycosidic linkage as an integral component of the lipopolysaccharide. By two methods of lipopolysaccharide purification (phenol-Mg^{++} precipitation and Pronase digestion), KDO was recovered exclusively in the lipopolysaccharide fraction at each stage of purification. Both methods yielded lipopolysaccharide having a KDO:heptose ratio of approximately 0.3. The properties of lipopolysaccharide-bound KDO are consistent with glycosidic linkage to the polymer. The carbonyl group of bound KDO is resistant to borohydride reduction. No significant destruction of KDO (measured by the thiobarbituric acid reaction after hydrolysis of the lipopolysaccharide) was observed after treatment of the intact lipopolysaccharide with $NaBH_4$; exposure to borohydride after release of KDO from lipopolysaccharide by mild acid hydrolysis resulted in complete loss of reactivity toward thiobarbituric acid. The linkage is resistant to alkali, but extremely labile to acid hydrolysis, as judged by appearance of reactivity in the thiobarbituric acid test and by liberation of free KDO, identified chromatographically. Figure 1 illustrates the time course of KDO release during acid hydrolysis. At 100°, the half-times of hydrolysis at pH 2.0, 3.0, and 3.4 were approximately 6, 9, and 12 min, respectively; at pH 4.5, the corresponding value was 45 min.

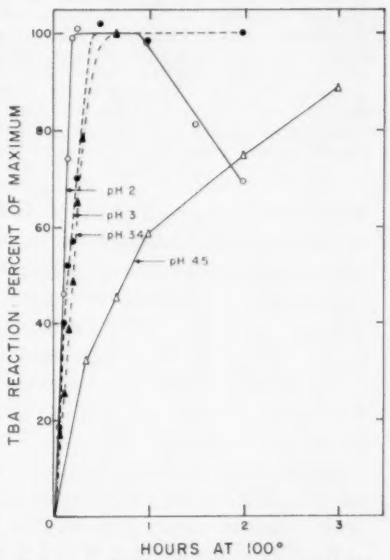


FIG. 1.—Release of lipopolysaccharide-bound KDO on acid hydrolysis. Suspensions of lipopolysaccharide (0.4 mg of carbohydrate per ml) were brought to the desired pH with H_2SO_4 (pH 2.0), acetic acid (pH 3.0 and 3.4), or ammonium acetate buffer (pH 4.5). The samples were heated in a boiling H_2O bath, and aliquots removed at the indicated times for analysis of TBA-reactive material. The value of total KDO obtained by hydrolysis in 0.02 *N* H_2SO_4 for 20 min was taken as 100%.

Isolation of polysaccharide-bound KDO: Hydrolysis of the insoluble mutant lipopolysaccharide at pH 3.4 and 100° resulted in quantitative release of polysaccharide and of KDO into the soluble fraction. Chromatography of the soluble hydrolysate on DEAE-cellulose with a gradient of pyridinium acetate, pH 5.3, gave the profile shown in Figure 2. Under the conditions used, free KDO was only partially adsorbed to the column, and was recovered in both fractions I and II. I and II contained no significant glucose, heptose, or phosphate; analysis showed a single component with the chromatographic and electrophoretic properties of free KDO. These two fractions accounted for 75 per cent of the thiobarbituric

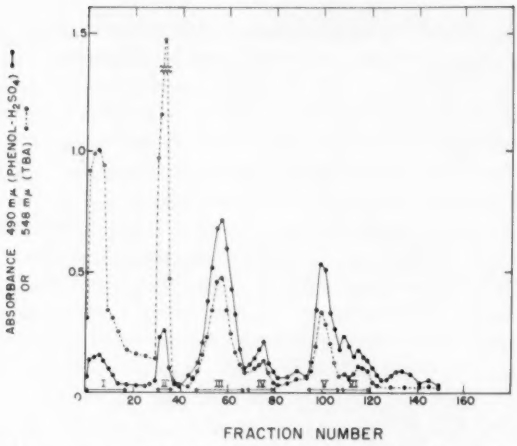


FIG. 2.—Chromatography of pH 3.4 hydrolysate on DEAE-cellulose. 25 ml of lipopolysaccharide equivalent to 187 mg of carbohydrate was adjusted to pH 3.4 with acetic acid, heated in a boiling H_2O bath for 30 min with stirring, chilled, and centrifuged. The insoluble residue was suspended in 0.01 *N* acetic acid, the 30 min hydrolysis procedure repeated 3 times, and the supernatant fractions pooled. At this point, over 98% of the carbohydrate was recovered in soluble form. The soluble fraction was diluted to 500 ml, adjusted to pH 8.2, and passed through a column of DEAE-cellulose (2.5 × 27 cm), previously equilibrated with 0.01 *M* Tris buffer, pH 8.2. Elution was begun with 200 ml of 0.02 *M* pyridinium acetate, pH 5.3; at fraction 14, a linear gradient of pyridinium acetate, pH 5.3 (1,000 ml 0.3 *M* into 1,000 ml 0.02 *M*) was applied. Total carbohydrate and KDO determinations were made on aliquots of alternate fractions. Concentrations of pyridinium acetate greater than 0.15 *M* interfere with the TBA reaction; the buffer was therefore removed by concentrating the sample to dryness *in vacuo* (37°) before carrying out the TBA reaction. Peak fractions were pooled as indicated and concentrated *in vacuo* at 37°.

acid-reactive material of the hydrolysate. The remaining 25 per cent was eluted at higher buffer concentrations in four peaks (III to VI) which also contained the phosphorylated heptose-glucose polysaccharide. The elution pattern of these thiobarbituric acid-positive fractions closely paralleled that of the total carbohydrate. Recoveries of total carbohydrate and thiobarbituric acid-reacting material were quantitative (97–105 per cent).

The thiobarbituric acid-positive material associated with the polysaccharide fractions was identified as KDO by paper chromatography of the isolated fractions after acid hydrolysis. Hydrolysis with 0.2 *N* HCl at 100° resulted in the gradual appearance of a thiobarbituric acid-positive spot corresponding to free KDO; no free KDO could be detected in the untreated fractions. Approximately 2 hr hydrolysis was required to liberate 50 per cent of the polysaccharide-bound KDO.

Further evidence that KDO is an integral constituent of the phosphorylated polysaccharide was provided by electrophoretic purification of the isolated DEAE fractions. The patterns obtained on high-voltage paper electrophoresis of fractions III–VI at pH 3.45 are shown in Figure 3. Duplicate samples were run on the same sheet and developed for total polysaccharide and for KDO. The pattern obtained with the two reagents was identical. Each DEAE fraction was separated into one major and several minor polysaccharide components, and every component gave a positive reaction with the thiobarbituric acid spray. Similar results were obtained on electrophoresis at pH 2.2, 4.2, and 6.0. Although the mutant polysaccharide

has been separated into at least 10 electrophoretically distinct components, no evidence of any KDO-negative fractions has been obtained.

Structural role of polysaccharide-bound KDO: Table 1 summarizes the analytical data on six of the purified fractions comprising 75–80 per cent of the total polysaccharide. All fractions contained glucose, heptose, and phosphate in addition to KDO. In general, the fractions showed only minor variations from an average glucose:heptose:phosphate ratio of approximately 0.6:1.2:1.0, except for the quantitatively minor fractions, IV-1 and IV-2, which contained substantially higher proportions of glucose. The ratio of KDO to heptose varied from 1:6 in fraction IV-2 to 1:28 in III-2. Ratios of 1:10 and 1:13 were obtained for the major fractions, III-1 and V-1, each of which accounted for 30–35 per cent of the total polysaccharide.

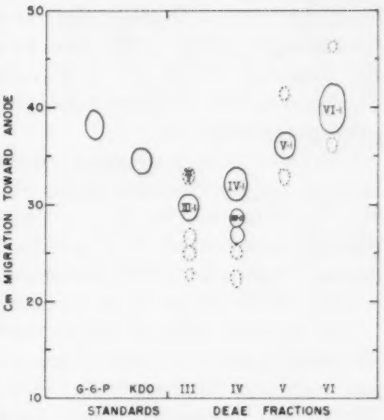


FIG. 3.—High-voltage electrophoresis of DEAE fractions. Approximately 50 μg of each fraction were applied in duplicate to Whatman 3MM strips (23 × 100 cm) and electrophoresed at pH 3.45 in pyridine:acetic acid:H_2O (1:10:69) for 1 hr at approximately 70 v/cm. KDO-containing areas were developed with the TBA spray.[22] For detection of carbohydrate, the paper was first sprayed with 0.02 *M* $NaIO_4$; after 3–5 min a spray of 5% $AgNO_3$ in H_2O was applied, followed by 0.5 NaOH in 95% ethanol. Carbohydrate containing spots appeared as brown areas on a tan background. After full development the background was decolorized by dipping in 3% $Na_2S_2O_3$.

The present evidence suggests that the bound KDO occurs at the reducing ends of polysaccharide chains. As indicated above, the purified polysaccharide fractions reacted directly in the thiobarbituric acid test without prior acid hydrolysis, and no increase in reactivity was obtained on such hydrolysis. The presence of a free KDO-carbonyl group was confirmed by borohydride reduction of the purified fractions (Table 1). Treatment with $NaBH_4$ resulted in complete destruction of KDO, as judged by disappearance of the characteristic spectrum in the thiobarbituric acid reaction. Failure to detect the

TABLE 1

ANALYSIS OF PURIFIED POLYSACCHARIDE FRACTIONS

Fraction	% of total polysaccharide	Molar Ratio* P	Heptose	Glucose	KDO	Heptose/KDO	Reducing value—moles (as glucose) per mole heptose
III-1	34.4	1.0	1.27	0.65	0.125	10.1	0.02
III-1 Reduced		1.0	1.28	0.68	0		
III-2	3.5	1.0	1.43	0.67	0.051	28.1	0.04
III-2 Reduced		1.0	1.42	0.65	0		
IV-1	2.6	1.0	1.32	1.07	0.184	7.1	0.03
IV-1 Reduced		1.0	1.36	1.09	0		
IV-2	0.8	1.0	1.26	1.30	0.203	6.1	0.03
IV-2 Reduced		1.0	1.30	1.47	0		
V-1	30.1	1.0	1.05	0.52	0.083	12.7	0.02
V-1 Reduced		1.0	1.04	0.51	0		
VI-1	5.5	1.0	1.06	0.54	0.063	16.8	0.03
VI-1 Reduced		1.0	1.07	0.54	0		

* Relative to phosphate.

Electrophoretically purified polysaccharide fractions (cf. Fig. 3) were analyzed by the procedures in *Materials and Methods*. Reduction was carried out with $NaBH_4$ (20 mg/ml) for 2 hr at 20°. Excess borohydride was destroyed by acidification with HCl, and borate was removed by concentration *in vacuo* from methanol.

formation of KDO hydroxamate after treatment with alkaline H_2NOH rendered ester linkage unlikely. Thin layer chromatography revealed trace amounts of acetic and long-chain fatty acid hydroxamates, but no KDO hydroxamate was found. The hydroxamate of 2-keto-3-deoxyheptonate (prepared from the lactone) was used as reference compound.

KDO was the only reducing end-group observed; no disappearance of glucose or heptose was detected after borohydride reduction of the purified fractions (Table 1). These results cannot be considered as conclusive evidence that all polysaccharide chains contain KDO as reducing end-group, since small amounts of terminal heptose or glucose could have escaped detection by the methods used. Efforts to detect such end groups by more sensitive techniques are in progress.

The reducing values of the polysaccharide fractions are very low (Table 1). If the reducing power of the end group were equivalent to that of glucose, the molecular weights of fractions III-1 and V-1 would be approximately 16,000 and 21,000, respectively. However, these values are not compatible with the behavior of the fractions on Sephadex G-25 and G-50, which indicated molecular weights close to 5,000. On the other hand, the low reducing values are consistent with reducing-terminal KDO, which gives about 10 per cent of the reducing value of glucose in the Nelson method. The minimum molecular weights calculated from KDO content, approximately 4,000 for III-1 and 5,300 for V-I, also agree well with the values predicted from chromatography on columns of Sephadex.

The presence of reducing-terminal KDO raises the possibility that the sugar acid is involved in the linkage of the polysaccharide to the lipid moiety of the intact lipopolysaccharide. The acid lability of the polysaccharide-lipid linkage is comparable to that of KDO glycosides. Figure 4 illustrates the release of polysaccharide and KDO into solution on hydrolysis of the insoluble lipopolysaccharide at pH 4.5 and 100°. Free KDO was separated from polysaccharide on DEAE-cellulose, and the free and polysaccharide-bound KDO determined separately. Bulk polysaccharide and polysaccharide-bound KDO were liberated at identical rates, in accord with the hypothesis that release of polysaccharide from the lipid results in the simultaneous appearance of reducing-terminal KDO. At pH 4.5, the rate of release of free KDO was 4-fold greater than that of polysaccharide-bound KDO; the half-times of hydrolysis were approximately 35 min and 130 min, respectively. At lower pH values, however, no significant difference was observed in the rate of liberation of the two fractions.

Discussion.—The present evidence indicates that the polysaccharide of the galactose-deficient mutant is composed of glucose-heptose-phosphate chains terminated at the reducing end by KDO. Chromatography and electrophoresis resulted in separation of the polysaccharide into fractions of different chain length, but of similar composition. The major fractions contained 0.5 mole of glucose and 0.8–0.95 mole of phosphate per mole of heptose, in addition to smaller amounts (0.06–0.10 mole) of KDO, present exclusively as reducing end-group. As yet little is known about the detailed structure of this core polysaccharide. However, some additional information has been obtained from studies[17] on a mutant of *S. typhimurium* which is unable to synthesize UDP-glucose. This organism is deficient in phosphoglucose isomerase, and forms an incomplete polysaccharide containing only heptose-phosphate and KDO. Similarly, heptose has been identified by other investi-

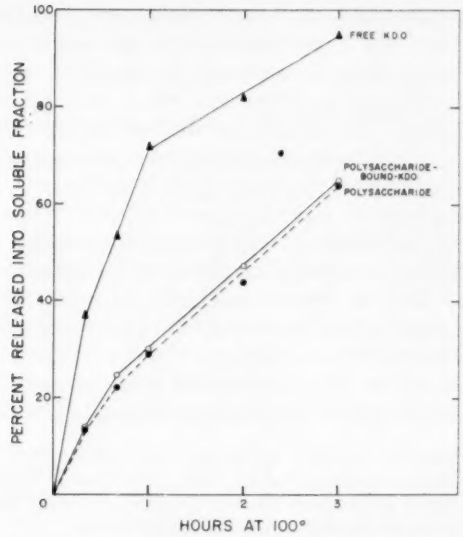
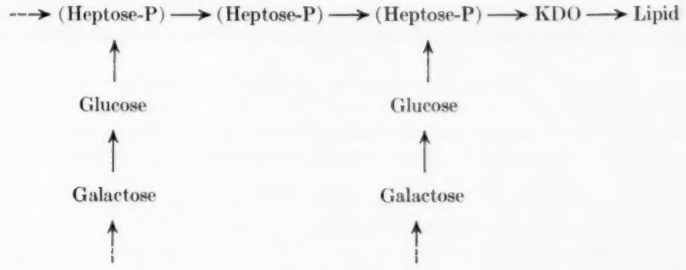


FIG. 4.—Release of KDO and polysaccharide from lipopolysaccharide at pH 4.5. Lipopolysaccharide was suspended in 0.025 *M* ammonium acetate, pH 4.5 to a final concentration of 1.3 mg of carbohydrate per ml. 2.0 ml aliquots were hydrolyzed at 100° for the times indicated, chilled, and centrifuged. The residues were suspended evenly in 2.0 ml H_2O by brief sonication, and both supernatant and residue fractions analyzed for total carbohydrate, total KDO, and TBA-reactive KDO. For separation of free and polysaccharide-bound KDO, 1.0 ml aliquots of the soluble fractions were diluted to 10 ml, adjusted to pH 8.2, and applied to 0.9 × 3 cm columns of DEAE-cellulose. 20 ml of 0.03 *M* pyridinium acetate, pH 5.3 were passed through the column and combined with the original effluent for determination of free KDO. Polysaccharide-bound KDO was eluted with 20 ml of 0.5 *M* pyridinium acetate, pH 5.3. 100% on the ordinate scale refers to the total amount of each fraction present in the original lipopolysaccharide, as determined in a separate experiment in which lipopolysaccharide was hydrolyzed at pH 3.4 (cf. legend for Fig. 2) under conditions known to give quantitative recoveries of each fraction.

gators[18, 19] as the major neutral sugar component in the polysaccharides of *E. coli* mutants lacking UDP-glucose pyrophosphorylase. The available evidence suggests a structure of the general type:



The nature of the linkage between heptose-phosphate residues in the backbone is not clear, nor is the site of linkage of glucose to the backbone known. However, evidence for the postulated linkage of galactose to glucose has been obtained from studies on the biosynthesis of the polysaccharide. A particulate enzyme system derived from the epimeraseless mutant has been shown to catalyze the transfer of

galactose from UDP-galactose to the 3-position of glucose residues in the endogenous lipopolysaccharide.[3, 20] The hypothesis that the polysaccharide is linked to the lipid moiety of the intact lipopolysaccharide through KDO is currently under investigation. The lipid has been shown to contain both β-hydroxy fatty acids[21] and glucosamine, either of which might provide sites for glycosidic linkage of KDO.

The KDO forming the reducing-terminus of the polysaccharide comprises only 25 per cent of the total KDO of the intact lipopolysaccharide. The site of attachment of the remaining fraction, which is released as free KDO on mild acid hydrolysis, has yet to be determined. It may occupy nonreducing terminal positions in the polysaccharide, but linkage to a component of the lipid has not been excluded. The possibility that all of the lipopolysaccharide-bound KDO is involved in the linkage of polysaccharide to lipid by poly-KDO chains, in a structure of the type: --→ Heptose → Heptose → KDO → KDO → KDO → KDO → Lipid appears to be unlikely, since at pH 4.5 the rate of hydrolysis of the polysaccharide-lipid linkage is appreciably slower than the rate of liberation of free KDO.

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HOST-DEPENDENT SYNTHESIS OF ALTERED DEOXYCYTIDYLATE HYDROXYMETHYLASE AFTER INFECTION OF ESCHERICHIA COLI WITH CERTAIN AMBER MUTANTS OF BACTERIOPHAGE T4,†*

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The amber (*am*) mutants of bacteriophage T4 are a class of mutants discovered by R. H. Epstein. Like certain strains of r II mutants of T4 they are "ambivalent,"[1] i.e., they are active in one bacterial strain but not in another. Infection of certain derivatives of *Escherichia coli* K12 (permissive hosts) leads to replication of the mutants, whereas infection of *E. coli* B is abortive (nonpermissive host).[2] In the case of infection of the latter host with certain amber mutants, no phage DNA is produced. The amber mutants *B269*, *N122*, *N55*, and *B21* used in this work fall into this category.[2]

Flaks and Cohen have shown that infection of *E. coli* with T-even bacteriophage results in the appearance of dCMP hydroxymethylase activity.[3] The catalytic function of this enzyme in the infected cell is the conversion of dCMP, a component of bacterial DNA, to deoxy-5-hydroxymethylcytidylate (dHMP), its counterpart in phage DNA.[3, 4]

In a previous communication from this laboratory,[5a] the failure of appearance of dCMP hydroxymethylase after infection of *E. coli* B with *am N122* (previously referred to as *am 122*) has been reported.[5b] The mutational site of *am N122* and those of the other mutants used in the work to be reported in this paper are in the same cistron (cistron 42).[2, 6a]

This paper deals with studies of dCMP hydroxymethylase formed in permissive hosts after infection with each of the mutant phages of cistron 42 and with *T4am*$^+$. From a comparison of the relative stability of these different enzymes with respect to temperature at a given pH, it has been concluded that the "mutant" enzymes[6b] represent alterations of the "wild-type" enzyme and that the host plays a specific role in determining the structure of the product of a mutant phage gene.

Materials and Methods.—The amber mutants used in this work had been derived from phage T4D by R. H. Epstein. Stocks of the mutants were grown in *E. coli* CR63. The wild-type T4 used in these experiments was a spontaneous revertant of *am N82* and will be referred to as T4*am*$^+$. T4*am*$^+$ was grown in *E. coli* B. In either case, the phages were purified as follows: bacterial debris was removed by centrifugation for 30 min at 5,000 × *g*, and the phages were collected from the supernatant liquid by centrifugation at 2°C for 1 hr at 20,000 × *g*. The phage pellets were covered with M9 medium[7] and stored in the cold to allow resuspension. The cycle of centrifugation and resuspension described above was repeated at least once. To ensure homogeneity, the stock suspension was again subjected to centrifugation at 5,000 × *g* for 30 min at 2°C. These suspensions are stable for several months without any indication of settling.

dCMP was purchased from the California Corporation for Biochemical Research. C^{14}-labeled formaldehyde was purchased from New England Nuclear Corporation. Tetrahydrofolic acid was prepared by the method of Blakley.[8]

Preparation of extracts from infected bacteria: Cultures of the respective strains of *E. coli* were grown under forced aeration at 37°C in the glycerol casamino acid medium of Fraser and Jerrel[9] to a concentration of 10^9 cells/ml. Aeration was stopped, and the cultures were allowed to stand at room temperature while aliquots were transferred into sterile flasks in a shaker bath at 37°C. L-tryptophan was added to a concentration of 10 μg/ml. After 2 min, 4 phage particles per bac-

terium were added, and 4 min later the addition of phage was repeated. The infections were carried out within 5–10 min after the bacterial cultures had reached the desired concentration. Aliquots of 5 or 10 ml were pipetted into liquid nitrogen 60 min after infection. The frozen pellets were stored at −20° C. Immediately before use in the assay, the cells were disrupted in a Hughes press.[10]

Concentrated extracts of *E. coli* B that had been infected with *am N122* were prepared as follows: aliquots of 100 ml of infected culture were mixed with 50 gm of frozen medium, and the cells were collected by centrifugation at 6,000 × *g* for 10 min at 2°C. The pellet was suspended in 10 ml of 0.02 *M* Tris buffer, pH 7.5, and the suspension was frozen in liquid nitrogen. The cells were disrupted in a Hughes press. The crude extracts obtained were used in the enzyme assays.

Enzyme assays: The assay for dHMP kinase has been described previously.[5]

dCMP hydroxymethylase: The reaction mixture (2.5 ml) contained: tris (hydroxymethyl) aminomethane buffer (chloride salt) or phosphate buffer (potassium salt) at the indicated pH values, 0.125 mmoles; dCMP, 10.5 μmoles; 2-mercaptoethanol, 0.250 mmoles; tetrahydrofolate, 2.25 μmoles; C^{14}-labeled formaldehyde, 5 μmoles with a specific activity of 1.2 μcurie/μmole; extract containing the material from 5×10^8 cells (in the case of *E. coli* CR63, C600, and CS^p infected with the amber mutants), 5×10^9 cells (in the case of *E. coli* B infected with *am N122*) and 2.5×10^8 cells (in the case of *E. coli* B or *E. coli* K12 infected with T4*am*$^+$). Before addition of enzyme the assay mixture was brought to the desired temperature. At 5, 15, 25, and 35 min after addition of enzyme, aliquots of 0.5 ml were transferred to tubes that contained 1 ml of water. Extracts of the three additional derivatives of *E. coli* B and of a nonpermissive derivative of *E. coli* K12 infected with T4*am*$^+$ or the mutants were tested for dCMP hydroxymethylase activity by incubation of the material from 5×10^8 cells with the reaction mixture described above at pH 7.5 for 40 min and at 25°C. The enzyme was inactivated by heating the vessels for 2 min in a boiling waterbath. The mixture was then applied onto a Dowex-1-8X formate ion exchange column (3.3 × 50 mm; 200–400 mesh resin). After adsorption of the material, the column was washed with 2 ml of water. The material was then eluted with 2 ml of 0.15 *M* formic acid onto a Dowex-50 W-4x ion exchange column (3.3 × 50 mm). The adsorption of the material on the Dowex-50 column was followed by elution of extraneous substances with 2 ml of 0.15 *M* formic acid. Elution of dHMP onto stainless steel planchets was then accomplished by application of an additional 5 ml of 0.15 *M* formic acid. In order to avoid complications that may arise from the effects of a specific buffer on the enzyme system, the determinations of pH optima were carried out on the incubation mixtures containing Tris buffer between pH 6.4 and 9, and on mixtures containing phosphate buffer between pH 6.4 and 8.5.

When comparisons were made of assays of enzyme produced by the wild-type phage or by the different mutants in a particular host, infections were carried out on aliquots of the same bacterial culture.

Results.—In studies on infection of *E. coli* CR63 by *am N122* Gilbert[11] showed that no dCMP hydroxymethylase activity could be detected when extracts prepared from such cultures were incubated with the substrate at pH 8 and 37°C. However, as will be shown in this communication, when the assay was performed at 25°C, dHMP was produced, and its concentration increased linearly with time of incubation with the enzyme. The results of such an experiment carried out at pH 7.4 and at three different temperatures are given in Figure 1*A*.

Assays of extracts of *E. coli* B infected with *am N122* performed under these conditions confirm the earlier report[5a] that no dCMP hydroxymethylase activity can be detected in such extracts. Furthermore, infection of either *E. coli* B or *E. coli* CR63 with T4*am*$^+$ results in the production of an enzyme that shows higher reaction rates with each elevation of temperature between 25 and 35°C. The Q_{10} in this range for the "wild-type" enzyme is 1.6–2.

These data suggest that infection of the permissive host CR63 with *am N122* results in the formation of an altered enzyme that is more labile than that produced after infection with T4*am*$^+$. The purpose of this communication is (*a*) to provide

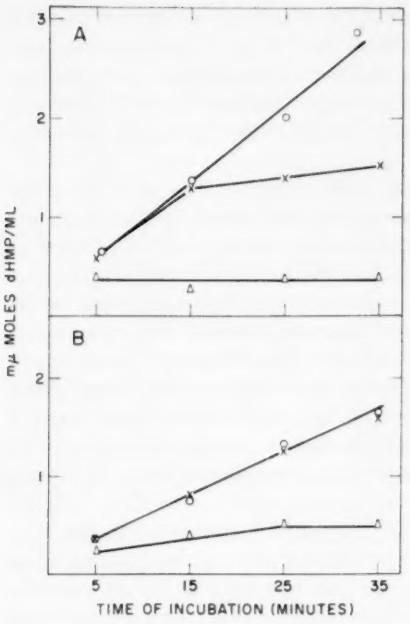

FIG. 1.—Formation of deoxyhydroxymethylcytidylate (dHMP) as a function of time of incubation of substrate mixture with enzyme extracts (CR63 infected with *am N122*) at 25°, 30°, and 35°C at pH 7.4 and pH 6.4. The amount of enzyme per ml is equivalent to 2 × 10^8 cells; temperature of incubation: O—O 25°C; ×—× 30°C; △—△ 35°C. pH of reaction mixture: *A*, 7.4; *B*, 6.4.

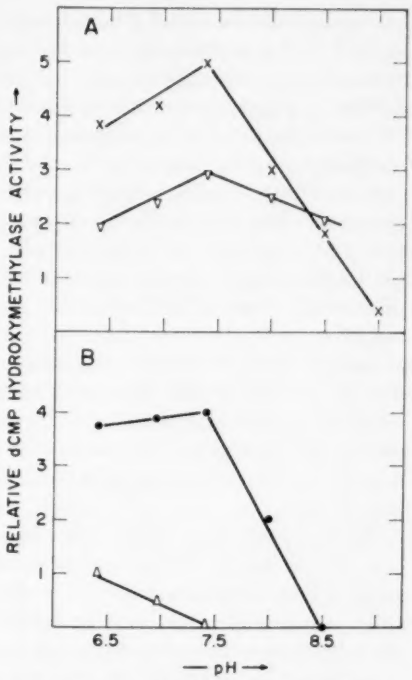

FIG. 2.—Deoxycytidylate hydroxymethylase activity as a function of pH at 25°C and 37°C. *A:* enzyme, extract of CR63 infected with T4*am*$^+$; temperature of incubation: ×—×, 37°C; ▽—▽, 25°C. *B:* enzyme, extract of CR63 infected with *am N122;* temperature of incubation: ●—● 25°C, △—△ 37° C.

evidence for this hypothesis by a comparison of some properties of mutant and wild-type enzymes, and (*b*) to provide evidence for the hypothesis of host participation in the formation of the mutant enzyme by a comparison of the relative stability of various mutant enzymes.

Comparison of some properties of the mutant enzyme (phage amN122 in CR63) with wild-type enzyme: The pH optimum was determined at 25°C and at 37°C for the "mutant" and "wild-type" enzyme. Inspection of Figure 2*A* reveals that the pH optimum for the "wild-type" enzyme is about 7.4, regardless of the temperature at which the measurements are made. However, there is a decrease in the ratio of dCMP hydroxymethylase activity at 37°C and 25°C, as the pH of the assay is increased from 7.5 to 8.5. Thus, at the higher temperature the effect of pH is more evident than at a lower temperature.

The data obtained from similar experiments with the "mutant" enzyme are presented in Figure 2*B*, and are interpreted in a similar fashion. The pH optimum at 25°C, a temperature at which the enzyme is relatively stable, is identical with that of the "wild-type" enzyme. However, at 37°C the "mutant" enzyme is

essentially inactive at pH 7.4, although a low level of activity is detected at pH 7 and 6.4. This apparent shift in pH optimum of the "mutant" enzyme with temperature is not thought to reflect a shift in the true pH optimum, if the latter is defined as primarily a characteristic of the active or binding site of the enzyme, but rather is considered to represent a protective effect of the hydrogen ions on the configuration of the protein.

Further studies on the relation of the stability of the enzyme with temperature: The hypothesis that the "mutant" enzyme loses activity by denaturation at high pH and at 37°C is borne out by the following experiments: (*a*) As previously stated, at pH 7.4 the mutant enzyme is inactivated at 37°C, although it is active at 25°C and production of dHMP is linear with time of incubation. No activity can be detected at 25°C, however, with the "mutant" enzyme that had been previously exposed to 37°C in the complete assay mixture and then chilled. (*b*) Even at low pH, linearity of dHMP production catalyzed by the mutant enzyme is not maintained over the 35 min period at 37°C, as it is at 25°C. (*c*) For assays of the mutant enzyme at 30° and pH 7.4, the rate of dHMP formation sharply declines after 15 min (Fig. 1*A*), whereas linearity with time is maintained up to 35 min at pH 6.4 (Fig. 1 *B*).

The foregoing data support the argument that dCMP hydroxymethylase produced after infection of *E. coli* with wild-type T4 becomes increasingly unstable as the pH is raised above 7.4. The degree of instability depends on the temperature. In the case of the enzyme produced after infection of CR63 with *am N122*, the temperature-dependent pH sensitivity is amplified.

No formation of dCMP hydroxymethylase in *E. coli* B after infection with *am N122* can be detected, even though a more sensitive enzyme assay has been used in these experiments than was used previously.[5a] In the revised method, analysis for dHMP was performed at several intervals during the incubation. The assays were carried out at 25°C and 37°C over a pH range of 6.4–8.5 with tenfold concentrated extracts (as compared to those prepared from CR63 infected with *am N122*). These attempts to find enzyme activity in the nonpermissive host, *E. coli* B, have been extended in a less rigorous manner to four other nonpermissive derivatives of *E. coli*, including one nonpermissive derivative of *E. coli* K12. In all instances, no enzyme activity has been found after infection with *am N122*.

In order to rule out the possibility that the lack of activity may result from the presence of an inhibitor in the nonpermissive host, a 1:1 mixture of extracts of CR63 and *E. coli* B, both infected with *am N122*, was tested for activity. No inhibition of the mutant enzyme was observed.

A further point of interest is that there is no difference in the amount of phage formed when CR63 is infected at 37°C with either *am N122* or T4*am*$^+$. At this temperature the mutant enzyme would be inactive in the assay *in vitro;* however, in the milieu of the intact cell it is active and functional at this temperature.

Studies of the action of other phage mutants in E. coli CR63: The amber mutants *N55*, *B269*, and *B21* isolated by Epstein have mutational sites in the same cistron as *am N122*.[6a] As an extension of these observations, Wiberg[12] has found that infection of *E. coli* B with *am N55* fails to result in the production of dCMP hydroxymethylase. The same is true for infection with *am B269* and *am B21*.

Three additional derivatives of *E. coli* B and one nonpermissive derivative of

E. coli K12 (kindly supplied by S. Penman) were tested with each of the four mutants and with T4*am*$^+$. dCMP hydroxymethylase activity was found only in extracts prepared from cultures that had been infected with wild-type phage, but not when the infection had been carried out with any of the mutants. In order to exclude the possibility that faulty injection was responsible for the lack of dCMP hydroxymethylase activity in any instance, all extracts were assayed for dHMP kinase activity. This enzyme had been induced in every case. Thus, one may conclude that, when *E. coli* B serves as host for any one of these mutants, no protein with appreciable dCMP hydroxymethylase activity is formed.

Similar studies have been made with the four phage mutants and with *E. coli* CR63 as the host; in each instance dCMP hydroxymethylase activity was observed when assays were carried out at 25°C and at pH 7.4. At 37°C little enzyme activity was found. However, when assays of the enzyme were carried out at 30°C, striking differences were observed in the enzyme activity in the extracts prepared from CR63 cells infected by different phage mutants. On the one hand, the typical curve was obtained relating dHMP formation to duration of the assay for the enzyme produced after infection of CR63 with *am N122*, i.e., synthesis of dHMP for the first 15-min period but little reaction thereafter (cf. Figs. 3*A* and 1*A*); on the other hand, after infection with any one of the other three phage mutants, the enzymes formed were quite stable throughout the entire 35 min assay period. Thus, although the enzymes produced after infection with *am N55*, *am B21*, and *am B269* could not be distinguished among themselves, they had stability characteristics markedly different from the enzyme formed after infection of CR63 with *am N122*.

Comparison of the enzymes formed in two additional permissive hosts after infection with the phage mutants: Studies similar to those described in the last section were carried out with two additional permissive hosts *E. coli* C600 and *E. coli* CS^P. Each of these bacterial hosts was infected separately with each of the four phage mutants, and the enzyme formed was assayed at 30°C and pH 7.4. The results are shown in Figures 3*B* and 3*C*, respectively. In contrast to the finding with *E. coli* CR63, dCMP hydroxymethylase formed after infection of C600 and CS^P with *am N122* is completely stable over the 35 min period of the assay at pH 7.4. However, the enzymes formed in either C600 or CS^P infected with *am N55*, *am B269*, or *am B21* show a pronounced instability during the assay at 30°C. Thus, the enzymes formed in C600 or CS^P after infection with a particular mutant have similar stability characteristics but, as judged by this criterion, they differ markedly from the enzyme formed in CR63 under identical conditions of infection.

Discussion.—The results of the experiments reported above indicate that the host participates in determining the structure of the product of the mutant forms of the phage locus controlling the synthesis of the enzyme dCMP hydroxymethylase. These mutations in cistron 42 lead to changes that prohibit the development of active enzyme in several nonpermissive hosts, including four derivatives of *E. coli* B and one of *E. coli* K12. In the permissive hosts altered enzymes are formed. Comparison of the stability of the "mutant" and "wild-type" enzyme under certain conditions of temperature and pH have shown that the mutant enzyme is more labile. This lability reflects some change in the primary structure of the enzyme.

The phage mutants used in this work have point mutations.[2, 6a] By analogy to

the findings of Ingram and others[13, 14] on the structure of abnormal hemoglobins and of Yanofsky and co-workers on protein A of the tryptophan synthetase system,[15] we may assume that a base change in the nucleic acid of the invading mutant phage has led to the production in the permissive host of an enzyme with a substitution of one amino acid for another. Yanofsky and co-workers[15, 16] have shown that a

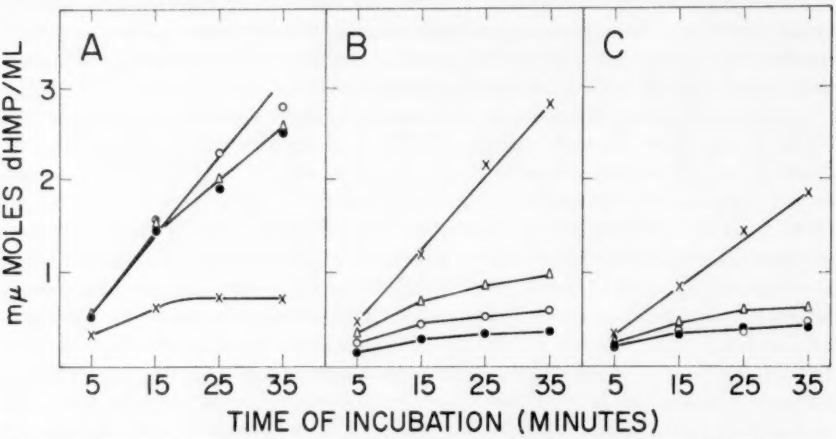

FIG. 3.—Formation of deoxyhydroxymethylcytidylate as a function of time of incubation at 30°C of substrate mixture with enzyme formed after infection of three hosts with several amber mutants. pH of reaction mixture, 7.35; phage (*am*) mutant: ●—●, *am N269;* ×—×, *am N122;* △—△, *am N55;* ○—○, *am N21;* bacterial host: *A*, CR63; *B*, C600; *C*, CSP.

single amino acid substitution in the polypeptide chain of protein A may greatly affect its stability characteristics.

The difference between the nonpermissive and the permissive hosts can be interpreted in a way similar to that proposed by Benzer and Champe[17] in connection with r II mutants of phage T4. Among the set of r II point mutants there are some that are unable to grow on the nonpermissive host, KB, but which can grow in permissive mutants of KB ("ambivalent" r II mutants). One such subset was analyzed further, and the results were interpreted as indicating that the mutated coding units spelled "nonsense"[18] in KB and "sense" (either original sense or missense[19]) in the host KB-3. In the r II system a direct comparison of the properties of the products made in the permissive host by the normal and the mutant genes could not be performed. Hence, these investigators could not distinguish between a reading of the code in the permissive host as "original" sense or missense.

In the case of the amber mutants we cannot determine at the present time whether the lack of appearance of dCMP hydroxymethylase activity in *E. coli* B represents lack of formation of the enzyme or the formation of an altered enzyme, either inactive or unstable under the conditions of assay we have used. From a comparison of the stability of the "mutant" and "wild-type" enzymes formed in the permissive hosts after infection by any of the phage mutants and by T4*am*$^+$, respectively, it seems likely that the mutant enzymes contain some alteration of their primary structure. Furthermore, as judged by the stability properties of the enzymes formed in different permissive hosts (cf. CR63 and C600), the specific alteration in the mutant enzyme produced by infection by the same phage may differ from host

to host. It is therefore tempting to speculate that the host cell contributes to the properties of the viral enzyme through the specificity of its own code translating machinery.

Summary.—The amber mutants of bacteriophage T4 are conditional lethal mutants. Infection of nonpermissive hosts, such as *E. coli* B, is abortive, and in the case of infection with the mutants used in this work no active dCMP hydroxymethylase is formed, whereas other phage-initiated enzymes are made. Infection of three derivatives of *E. coli* K12 that permit replication of the mutants results in the formation of enzymes that show differing degrees of temperature sensitivity. The enzymes formed after infection of the three permissive hosts with each of the four mutants (*am B269*, *am N122*, *am N55*, or *am B21*) were tested for relative heat stability. In any host certain differences were found in the stabilities of the enzyme formed after infection with different mutants. A comparison of the relative temperature sensitivity of the enzyme induced by one mutant in the different hosts revealed host dependence with respect to the nature of the protein formed. The evidence presented is in accord with the hypothesis that the mutation in the phage genome gives rise to nonsense or unacceptable missense in *E. coli* B, and to acceptable missense in the permissive hosts.

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† The following abbreviations are used: DNA, deoxynucleic acid; dCMP and dHMP, the 5′ monophosphates of deoxycytidine and deoxy-5-hydroxymethylcytidine; Tris, tris (hydroxymethyl) aminomethane.

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[6b] The mutant enzyme refers to any one of the enzymes produced by the infection of one of the permissive hosts with bacteriophage *am N*122, *am N*55, *am B*269, or *am B*21; the wild-type enzyme refers to the enzyme produced by infection of either the permissive or nonpermissive host by the wild-type phage, T4*am*.$^+$

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*ON RELATIVE VIABILITY AND REPLACEMENT**

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In a recent issue of these Proceedings, Falk and Rahat[1] have suggested that intraculture competition and replacement, rather than relative viability, can account for the observed frequencies of flies of different genotypes in experimental cultures. Specifically, this suggestion has been put forward to explain the higher frequency of flies heterozygous for irradiated than for control chromosomes in certain of my experiments.[2, 3] In reference to studies on the X-chromosome, Falk and Rahat suggest that some males hemizygous for an irradiated chromosome fail to develop and that these missing males are "replaced" by females heterozygous for this same chromosome.

Replacement can take two forms. If flies of one genotype are missing from a somewhat overcrowded culture, the remaining classes may compete for the unused medium. This is the same situation that obtains when some vials or culture bottles have slightly more or slightly less culture medium than others. The outcome of this competition gives us a measure of the relative viabilities of flies possessing different genotypes. "Viability," in this sense, is a complex measure involving hatchability of eggs, survival of larvae and pupae, competitive ability, and developmental rates (since counts of adults are terminated prior to the emergence of second-generation flies). The complexity of this measure of viability was pointed out by Wallace and King[4] some dozen years ago and again by Wallace;[5] its complexity is a desirable feature since viability in these studies is measured in an effort to estimate fitness, a phenotypic trait comprising many components.

There remains, however, another kind of replacement that could seriously alter the outcome of studies on the viability effects of irradiated X-chromosomes. One can imagine that within each test culture there exists a micro-niche shared jointly, and exclusively, by wild-type male larvae and female larvae heterozygous for the same wild-type chromosome. If this were true, death of male larvae carrying an irradiated X-chromosome would release a larger share of this hypothetical micro-niche to heterozygous females. Thus, there would be a tendency for mutations deleterious to males to appear beneficial to females.

A search for this second kind of replacement has been carried out by comparing the numbers of flies of various genotypes emerging in comparable lethal and nonlethal cultures. Approximately 5,000 control and irradiated X-chromosomes have been tested (Wallace[2] and unpublished data); each test involved a single half-pint culture. Three levels of irradiation were used in these X-chromosome studies (500 r, 1,000 r, and 2,000 r), and for each there was an appropriate control. These cultures were randomized and coded at the time they were prepared. A total of 77 lethals were recovered among the irradiated material; for comparative purposes we have chosen these and 77 nonlethals (the nonlethal X-ray cultures immediately following each lethal on the decoded summary data sheet) for analysis. Lethals have been chosen since the absence of wild-type males in these cultures should release the entire micro-niche to heterozygous females, if such a micro-niche exists.

The pertinent data have been summarized in Tables 1 and 2; the crosses involved correspond approximately to Falk and Rahat's Series I and II, respectively. It can be seen that the missing males are indeed replaced to some extent; this has consistently been my experience in studies on *D. melanogaster*, but a similar replacement does not seem to occur in *D. pseudoobscura* (Dobzhansky, personal communication). The important fact, however, is that heterozygous females carrying an irradiated chromosome do not possess a unique "replacement" ability. The lethal

TABLE 1

	No. of cultures	+♂♂	B♂♂	+/B♀♀	B/B♀♀	Total	%+/B♀♀
Lethal	45	0.6	58.9	79.0	59.7	198.2	57.0
Nonlethal	45	70.4	52.6	73.7	52.1	248.7	58.6

The average numbers of flies of different classes observed in lethal and comparable nonlethal cultures in which Basc males and females heterozygous for Basc and an irradiated, wild-type X-chromosome were parents.

TABLE 2

	No. of cultures	+♂♂	B♂♂	+/+♀♀	+/B♀♀	Total	%+/+
Lethal	32	2.3	62.3	80.9	84.9	230.4	48.8
Nonlethal	32	65.9	50.3	66.4	63.5	246.2	51.1

The average number of flies of different classes observed in lethal and comparable nonlethal cultures in which wild-type males and females heterozygous for Basc and an irradiated, wild-type X-chromosome were parents.

heterozygotes have somewhat impaired viabilities; this type of information has often been used as evidence for the incomplete recessivity of newly induced lethals. To retain the "replacement" hypothesis one now needs to propose that every recessive lethal is simultaneously a dominant "replacement" suppressor; this makes the hypothesis unacceptably complicated.

There is no evidence in the data presented here that replacement of the sort that causes spurious ratios (more precisely, ratios leading to spurious estimates of relative viabilities) occurs in the tests involving the X-chromosome. Evidence of a similar sort relevant to the second chromosome studies has been presented previously (Wallace,[3] p. 541). Replacement that does not alter the relative frequencies of remaining classes of flies does not affect the validity of our experimental procedures.

Suggestions on the interpretation of experimental data can be evaluated in two ways: one can examine closely those facets of the experimental data that bear specifically on the suggestion; this we have done above. One can also ask whether the suggestion possesses sufficient merit so that it can be applied impartially to all relevant situations.

Falk and Rahat, for example, observed in crowded cultures an excess of flies carrying an irradiated chromosome; this excess seemingly vanished under near-optimal conditions. (The statistical significance of the apparent deficiency of radiation heterozygotes under the near-optimal conditions was not given.) They state explicitly that the more intraculture competition is relaxed, the more pronounced is the deleterious effect of radiation. Previously, however, Muller and Falk[6] used 15 pairs of flies as parents in each culture vial because, as they said, small genetic influences on viability are expressed to a more marked degree when competition is increased by crowding. The replacement hypothesis may be attractive in explaining away *heterotic* effects because these are unexpected; would

it be equally attractive, however, if it were to be used in an effort to explain away apparent *deleterious* effects of radiation? Unless experiments on the viability effects of radiation are to take on a "heads I win; tails you lose" character, explanatory hypotheses must be equally acceptable under both circumstances.

* Contribution no. 442, Department of Plant Breeding, Cornell University. Work reported here was done under contract AT-(30-1)-2139, U.S. Atomic Energy Commission.

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INTERMOLECULAR FORCES AND BIOLOGICAL SPECIFICITY

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Many biochemical reactions are highly specific in that the components entering them are brought close together, properly oriented and channeled off in a synchronized interplay of several molecules, enzymes usually being involved in the process. In this paper we are concerned about the more specialized question of the cause of specific association of two molecules and, by inference, the specific association of cellular components and of entire cells with each other. These issues of specific interaction are important in biology and in understanding the formation of molecular van der Waals crystals.

On the one hand, a large class of intermolecular interactions of high biological specificity arises from the interaction of complementary sets of charges distributed over the corresponding contact points of the interacting, sterically compatible molecules (e.g., hydrogen bond complementarity in DNA). On the other hand, London-van der Waals forces, or more generally all kinds of charge fluctuation forces,[1] come into the foreground if static charges, dipole, etc., moments of the interacting molecules are either negligible or compensated for by gegenions. London forces are due to the interaction between adjacent polarizable molecules or molecular groups; the terms "dispersion forces" and "correlations" are often used instead of "charge fluctuation interaction." These interactions may provide an understanding of phenomena implying a high degree of biological specificity. Two different types of mechanisms account for specificity of charge fluctuation interactions:

(1) *Complementarity:* The general attraction between neighboring sets of atoms implies that structures which fill out space are energetically advantageous; Pauling calculated the contributions toward their stability.[2]

(2) *Self-recognition:* Charge fluctuation forces have the remarkable property[3, 4]

of favoring association of identical molecules as nearest neighbors (cf. Fig. 1) when intermingled with other molecules immersed in a liquid.

In principle, this may be understood as due to the fact that the London energy $\Delta A_{I\,II} = (A_{I\,II})_R - (A_{I\,II})_\infty$ between a pair of molecules I, II is proportional to the product $-\alpha_I\alpha_{II}$ of their polarizabilities. When two identical (globular) molecules II, II want to get together as nearest neighbors, some medium molecules (we group them into aggregates I of the size of the macromolecules II) must give way, resulting

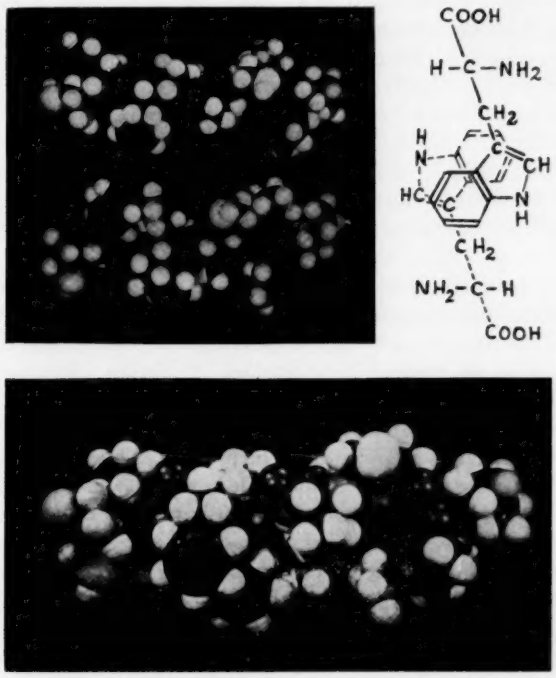

FIG. 1.—Two identical peptide sequences, one under a veil, one over it. The upper picture shows them separately, while in the lower picture they are close enough to interact. Each side chain interacts specifically with the identical partner. The total interaction has accordingly a very high degree of specificity (cf. question 3). The line drawing shows the orientation of the side chains when in specific London interaction.

in two fewer I, II pairs and one added I, I pair. This amounts to a rearrangement energy, well known in the theory of mixtures, $\Delta_4 A_{I\,II} = \Delta A_{I\,I} + \Delta A_{II\,II} - 2\Delta A_{I\,II}$, proportional to $-\alpha_I\alpha_I - \alpha_{II}\alpha_{II} + 2\alpha_I\alpha_{II} = -(\alpha_I - \alpha_{II})^2 \leq 0$. Thus, a preferential association of molecules with identical polarizabilities α results. As a prototype of the more general situation, we compare the preference of an association of

I	I	I	I	I	I			I	I	I	I	I	I
I	II	II	I	III	I		with	I	II	I	II	III	I
I	I	I	I	I	I			I	I	I	I	I	I

The rearrangement free energy is $\Delta A_{II\,II} - \Delta A_{II\,III} + \Delta A_{I\,III} - \Delta A_{I\,II} = {}^1/_2(\Delta A_{II\,II} + \Delta A_{III\,III} - 2\Delta A_{II\,III}) + {}^1/_2(\Delta A_{II\,II} + \Delta A_{I\,I} - 2\Delta A_{I\,II}) - {}^1/_2(\Delta A_{III\,III} + \Delta A_{I\,I} - 2\Delta A_{III\,I})$ which is usually negative.

Before discussing specificity of charge fluctuation interactions, it may be appropriate to remind ourselves of the interplay of the variety of forces which is expected to accompany such interactions. We presume that an equilibrium is established between (*a*) specific attractive forces due to charge fluctuations, and (*b*) nonspecific static electric interactions modified by a Debye-Hückel-Onsager layer of gegenions. The latter depend largely on the ionic constitution of the medium, changes of which essentially determine the balance between these interaction forces (*a*) and (*b*) (cf. H. C. Hamaker and the Dutch School[5]). As regards the net charge contributions, (*b*) amounts to a repulsion between identical molecules in the absence of gegenions. As regards the dipole contributions, both (*a*) and (*b*) have similar effects tending to orient the interacting molecules toward each other. The intermolecular distance at which these interactions operate is that small equilibrium distance between (*a*) and (*b*) unless, in the case of negligible (*b*), the van der Waals repulsions take their place.

The interplay between attractive and repulsive forces, rather than being static, may change with ionic conditions of the medium, perhaps periodically with a period marking the time required for completion of the biochemical process.

For the present, we neglect consideration of charge exchange interactions. Furthermore, as regards the differential interaction of different molecules with water, we consider only London forces. With the calculations of Sinanoglu, of Firsov, and of Mulliken, it will eventually be possible to handle the full problem.

Charge Fluctuation Interactions.—A molecule interacts with its neighbor molecules by virtue of the electron and proton charge distributions on the molecules. Charge fluctuation forces arise when fluctuations of charge distribution in a molecule are brought about by quantum mechanical motion, largely due to zero point energy ${}^1/_2\hbar\varpi$, or by thermal motion of energy kT. As regards charge fluctuations, a statistical preference of the energetically most favorable charge distributions results in "correlations" in adjacent molecules and in attraction.

With the quantum mechanical or thermal charge fluctuations of electronic oscillators, one has the London-Eisenschitz-Wang force, or its generalization which includes a Maxwell-Boltzmann distribution of electrons jumping between thermally accessible excited states (of which there are, however, only rare instances among the biologically interesting molecules). With fluctuations in charge distribution associated with molecular vibrations, the interaction energy is very small, unless there is proton motion over labile H bond bridges. If that occurs or if there is Brownian motion of protons on the surface of larger molecules, one has Kirkwood-Shumaker forces which may contribute considerably to the interaction (cf. refs. 1 and 3). The proton mobility in water surrounding some nucleic acids and proteins will be very large due to almost simultaneous charge transfers within the H bonds of domains of structuralized water[6]—domains which are characteristic of the type of hydrated molecules of nucleic acid or protein. Whatever the influence of the ionic medium on charge fluctuation interactions, it should be the same on two identical molecules; self-recognition is thereby not disturbed.

If no excited states are in thermal reach, and a molecule is represented by a single

electronic oscillator, isotropic and three-dimensional, of frequency ϖ, the calculation[1] follows: the oscillators of the molecules I, II are coupled by electric dipole-dipole interactions which bring about normal mode frequency displacements (and corresponding level shifts) containing $\pm R^{-3}$ and $-R^{-6}$ terms. The normal mode frequencies $\omega_l = \omega_x^{\pm} = \omega_y^{\pm} = \varpi(1 \pm \alpha/2R^3 - \alpha^2/8R^6 \pm \ldots)$, $\omega_l = \omega_z^{\pm} = \varpi(1 \mp \alpha/R^3 - \alpha^2/2R^6 \mp \ldots)$ contribute a zero point energy each, and the sum of these zero point energy shifts gives an attraction energy which in the more general case of unequal molecules is

$$\Delta A_{\mathrm{I\,II}} = -{}^3/_2 R^{-6}\alpha_{\mathrm{I}}\alpha_{\mathrm{II}}\hbar\varpi_{\mathrm{I}}\varpi_{\mathrm{II}}/(\varpi_{\mathrm{I}} + \varpi_{\mathrm{II}}) \ (\textit{London formula}) \tag{1}$$

where ϖ_{I}, ϖ_{II} are the uncoupled frequencies of the oscillators, and α_{I}, α_{II} their static polarizabilities.

With excited oscillator states, one may have the situation of one excited molecule approaching another identical but unexcited molecule.[7] Sharp resonance brings about interaction energies $\propto \pm R^{-3}$. The statistical Boltzmann averaging over excited states results in the partition function

$$Z = \textstyle\sum_{nl=0}^{\infty} \exp\left(-\sum_{l=1}^{6}(n_l + {}^1/_2)\hbar\omega_l/kT\right) \doteq \prod_{l=1}^{6}(kT/\hbar\omega_l)$$

in the classical limit of low frequency oscillators. The interaction Helmholtz free energies are for two identical molecules, and in general

$$\Delta A = kT\textstyle\sum_{l=1}^{6}(\ln\omega_l - \ln\varpi) = -3R^{-6}\alpha^2 kT$$

$$\Delta A_{\mathrm{I\,II}} = -3R^{-6}\alpha_{\mathrm{I}}\alpha_{\mathrm{II}}kT. \quad (\textit{Classical formula}) \tag{2}$$

The Kirkwood-Shumaker contributions are notable here. Even though kT is usually small, they are important because of their high effective polarizabilities.

The actual situation implies participation of both classical and quantum frequency ranges; again $\Delta A_{\mathrm{I\,II}} \propto R^{-6}$ and retardation is irrelevant. In this general case, we represent the molecules by *many oscillators* which have their natural *frequencies distributed* over classical and quantum regions, and whose spatial orientations show *anisotropies*. The calculation involves an evaluation of the R dependent normal mode frequencies of molecule pairs and of the partition function of a Boltzmann distribution over the quantum states of those harmonic normal mode oscillators of a molecule pair I, II, and establishes how, in the many oscillator molecules I, II, the interaction depends on the distribution of static oscillator polarizabilities α_l over the diverse quantum frequency regions ϖ_l (no sharp resonances), and over the direction cosines u_{lx}, u_{ly}, u_{lz} of oscillator orientations characterizing their anisotropies (l numbers the linear oscillators in the molecule I, $l = 1, 2, \ldots N_{\mathrm{I}}$; $\alpha_l = f_l\epsilon_l^2/m_l\varpi_l^2$; $m\mu$, ν number x, y, z). The Helmholtz free energy of such a distribution contains an R dependent term

$$\Delta A_{\mathrm{I\,II}} = -{}^1/_2 kT \ \mathrm{trace} \textstyle\sum_{s=-\infty}^{\infty}(\mathfrak{W}_{s\mathrm{I}}\mathfrak{W}_{s\mathrm{II}}) \tag{3}$$

where s runs through all integers and where the 3×3 matrix "$\mathfrak{W}_{s\mathrm{I}}$," i.e.,

$$\mathfrak{W}_{s\mu\nu\mathrm{I}} = -R^{-3}\sum_{l=1}^{N_{\mathrm{I}}}\frac{\alpha_l}{1 + (2\pi kT/\hbar\omega_l)^2 s^2}\begin{pmatrix} u_{lx}^2, & u_{lx}u_{ly}, & \sqrt{2}u_{lx}u_{lz} \\ u_{ly}u_{lx}, & u_{ly}^2, & \sqrt{2}u_{ly}u_{lz} \\ \sqrt{2}u_{lz}u_{lx}, & \sqrt{2}u_{lz}u_{ly}, & 2u_{lz}^2 \end{pmatrix} \tag{4}$$

is the dynamic polarizability $\alpha(\omega)$ taken at imaginary $\hbar\omega = 2\pi ikTs$,

$$\alpha(\omega) = \sum_l \alpha_l \vec{u}_l \vec{u}_l / [1 - (\omega/\varpi_l)^2]. \tag{4a}$$

The London formula (1) and classical formula (2) are readily obtained as special cases of (3) and (4) (cf. *Phys. Rev.*[3]). The neglect of quadrupole and octupole terms fortunately represents intermolecular interactions fairly well.

As the $\Delta A_{\mathrm{I\,II}}$ of (1), (2), and (3) are bilinear in α or in $\mathfrak{W}$,

$$\Delta_4 A_{\mathrm{I\,II}} = -{}^1/_2 kT \sum_{s=-\infty}^{+\infty} \sum_{\mu,\nu=1}^{3} \{\mathfrak{W}_{s\mu\nu\mathrm{I}} - \mathfrak{W}_{s\mu\nu\mathrm{II}}\}^2 \leq 0 \tag{5}$$

which again implies that identical molecules tend to associate as nearest neighbors. This is a sum over s (cf. Fig. 2), not over l.

The following questions often arise:

1. How large is the *London energy* $\Delta A_{\mathrm{I\,II}}$ between a *pair of molecules?* For small molecules this London interaction energy[3]

$$\Delta A_{\mathrm{I\,II}} \approx -{}^3/_4 R^{-6} (\sum_l \alpha_l)^2 \hbar \langle \varpi_l \rangle = -{}^3/_4 R^{-6} e^4 m^{-2} (\sum_l f_l)^2 \hbar \langle \omega_l \rangle^{-3} \tag{6}$$

is estimated from experimental single term dispersion curves which yield the dynamic polarizability α in the visible and near UV, this is $\approx \sum_l \alpha_l$ of the electronic oscillators, by (4a); and they yield an average oscillator frequency $\langle \varpi_l \rangle$ which is $\approx$ ionization energy$/\hbar$.[1] An alternative experimental determination of $\alpha_l = \alpha_l(\omega_l, \vec{u}_l)$ from absorption in the all important far and extreme UV is not yet in reach. An estimate of (6) is possible on the basis of the Thomas-Reiche-Kuhn sum rule which limits $\sum_l f_l$ essentially to the number of electrons in the valence shell (cf. Kronig and Kramers[8]). The resulting interaction energies $\Delta A_{\mathrm{I\,II}}$ are of the order of several kT. For a pair of interleaving cyclic or polycyclic side chains the effective intermolecular distances R are comparatively small, and the interaction energies may be of the order of $10kT$. Recent work on hypochromism[9] and other effects has furnished similar estimates on $\Delta A_{\mathrm{I\,II}}$.

2. The molecular average of the polarizabilities of unlike molecules may sometimes differ only by a moderate fraction. The quantity upon which the *rearrangement free energy* depends, i.e.,

$$\Delta_4 A_{\mathrm{I\,II}} \approx -{}^3/_4 R^{-6} (\sum_{\mathrm{I}} \alpha_l - \sum_{\mathrm{II}} \alpha_l)^2 \hbar \langle \omega_l \rangle \leq 0$$

is then only the square of that fraction times $\Delta A_{\mathrm{I\,II}}$ (1). How can rearrangement free energies still be significant for biological specificities? Actually, the rearrangement free energy (5) depends on differences between molecule I and molecule II, not of scalar polarizability averages but of the distribution of α_l over (u_{lx}, u_{ly}, u_{lz}) and over ϖ_l. Anisotropy of the structure of the molecule I implies that the representative dipole oscillators l and $\mathfrak{W}_{s\mu\nu\mathrm{I}}$ are anisotropically distributed. The effect of differences in frequency distribution of the polarizabilities of molecules I, II on the rearrangement energy $\Delta_4 A_{\mathrm{I\,II}}$ (5) is illustrated in Figure 2 which shows three typical curves whose superposition, with coefficients characterizing molecule I, gives $-\mathfrak{W}_{s\mathrm{I}}$, and similarly $-\mathfrak{W}_{s\,\mathrm{II}}$. The size of the term (5) is therefore expected to be more closely of the order $-{}^1/_2 kT \Sigma_s \Sigma_{\mu,\nu} (\mathfrak{W}_{s\mu\nu\mathrm{I}})^2$ rather than only a few per cent of it.

3. How is it that, even though only a moderate amount of energy is involved in $\Delta_4 A_{\mathrm{I\,II}}$, there may be a high *degree of specificity* which does not depend solely on the

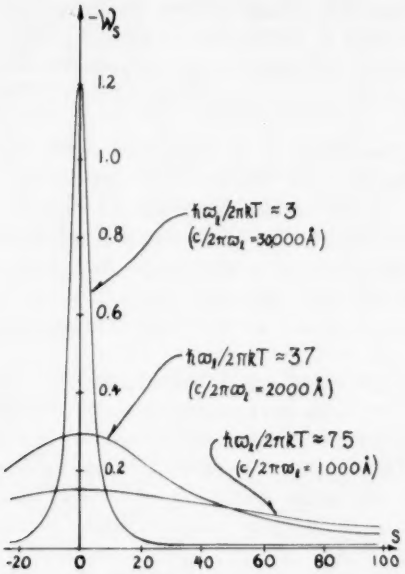

FIG. 2.—Molecular interactions are characterized by the value of $-\mathcal{W}_s$ for the molecules. Apart from a constant, $-\mathcal{W}_s$ is the dynamic polarizability (cf. equations 4 and 4a) analytically continued into the purely imaginary domain $\omega = i\,s\,2\pi kT/\hbar$. The graph shows $-\mathcal{W}_s$ as a function of the integers for different values of $\bar{\omega}_l$ (the frequency of the major contributing oscillator strengths). $-\mathcal{W}_s$, according to (4), is made up of a superposition of contributions from those spectral regions important for the molecular interactions (in this example three regions), thus providing for a three-parametric distribution $-\mathcal{W}_s$. Molecules differ in the amounts they contribute in each spectral region. The graph does not show the effects of anisotropy on the specificity of interaction.

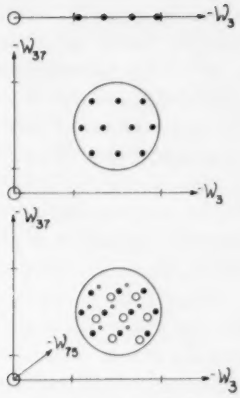

FIG. 3.—Comparison is made of the degree of specificity in the case of one-, two- and three-parametric distributions of molecular types in a manifold of molecules. By equation (5), the square of the distance between neighboring points represents the thermal noise; the square of the entire interval (length of line segment, diameter of circle, diameter of sphere) represents the maximum signal, equation (5), in the manifold of molecular pairs. The maximum signal is chosen to be the same in each graph. The number of points in each graph indicates the number of distinguishable molecular types. This number characterizes the degree of specificity, greatest in the case of the most numerous (three) parametric distribution.

magnitude of $\Delta_4 A_{\text{I II}}$? $\Delta_4 A_{\text{I II}}$ (5) is a sum of negative definite terms, each of which contributes toward the preferential association of identical molecules. The degree of specificity of discrimination between pairs of molecules out of a given manifold of molecule types may be represented by the number of cases (types of pairs) whose signal to noise ratio (ratio of London rearrangement energy to thermal energy) $|\Delta_4 A_{\text{I II}}|/kT > 1$, compared to the total number of cases for which it has any value. This degree of specificity depends not only on the ratio of the size of the signal to that of the noise in that manifold of molecule pairs, but also on the effective number of parameters (oscillator frequency regions and oscillator orientations) on which the distribution of $\mathcal{W}_{s\mu\nu\text{I}}$ over the manifold of molecules depends. Differences between $\alpha_{l\text{II}}$ and $\alpha_{l\text{III}}$ either in the classical region, in the UV, or in the extreme UV, and the differences in anisotropy of α in these regions cause significant differences between

$\mathfrak{W}_{s\mu\nu\mathrm{I}}$ and $\mathfrak{W}_{s\mu\nu\mathrm{II}}$. If, under those conditions, the set of molecule pairs (all combinations of closely adjacent pairs) has an expectation value for signal-noise ratio $\langle|\Delta_4 A_{\mathrm{I\,II}}|/kT\rangle_{AV}$ substantially greater than 1, the number of cases I, II for which $\langle|\Delta_4 A_{\mathrm{I\,II}}|/kT\rangle_{AV} > 1$ is very much greater than the number of cases for which it is <1, simply because of the multiparametricity of the distribution (Fig. 3); this means a high degree of specificity.

4. Must molecules be identical to be capable of specific interaction? No, even for the interactions due to London forces which arise between identical molecules, identity of structure is a sufficient, not a necessary condition. Molecules would have to be nearly identical only with respect to their distribution of polarizabilities.

5. What time is needed in effecting specific association? Brownian motion shuffles all molecules and so brings identical molecules together before specific attachment sets in. Brownian motion is rapid; specific association may occasionally be speeded up by zip action.

6. Given a pair of identical molecules with many side chains, how does the energetically most favorable attachment occur and how specific is it? Speaking for London interactions only, a pair of linear chains may interact as shown in Figure 1; identical α helixes may zip together as shown in our paper II[3] (and intertwine[10]). The side chains should be identical; the larger secondary or tertiary structures might, however, be quite different or occasionally be mirror-related,[10] as they contribute little to the interaction.

7. What is the role of the specificity of London-van der Waals forces in the formation of *van der Waals crystals?* In situations in which mainly London-van der Waals forces are of importance (compared with hydrogen bonding or ionic or polar interactions), the specific anisotropic London interactions tend to influence crystallization and contribute toward the purity of the crystal formation. The orientation effect as well as the selection of identical molecules as nearest neighbors (self-recognition) is, however, often counteracted by the tendency toward closest packing (complementarity) as the shape of molecules is usually far from spherical.[11] On the one hand, considering many identical small molecules, the structure determining factor usually is the tendency to arrange these molecules (perhaps in a regular combination with other molecules) so that there is a minimum of empty interstices. The resulting structure will, however, usually not be the one which offers the best mutual molecular orientations as suggested by the above considerations. On the other hand, consider identical macromolecules, helical or other, in a medium composed of small molecules; the form and character of the side chains of the macromolecules is usually so complex that space filling can only be achieved by small medium molecules filling interstices, usually in a random way. The mutual arrangement of the macromolecules may then be primarily determined by the above specificity considerations.

8. In which biological phenomena may specific London interactions be of importance? Phenomena which imply self-recognition[12] are basic for the workings of biological systems. Muller[13] has pointed out the importance of the accurate pairing mechanism of chromosome sections at synapsis, and even inverted synapsis, of chromosomes. Presumably molecules (perhaps mucoproteins or saccharides), characteristic of the respective chromosome sections, which populate the outermost layers of the chromosomes, specifically pair up with their partners in the fashion

indicated in Figure 1. A related phenomenon may be the organ specific association of cells,[14] so important for embryonic development and for specific inborn synaptic connections of neurons.[15] Such specificity may play an important role in protein and nucleic acid synthesis through collection of an identical replica from monomers.[13]

It is clear that biological specificity implies a great many mechanisms and different types of forces (as does crystallization). In order to clarify the role of the charge fluctuation forces, however, we set ourselves to the task of determining how far one may get in understanding biological specificity by considering only contributions from these charge fluctuation interactions.

Credit for this work is mostly due to Dr. Jerrold M. Yos, to Dr. William L. Bade, and to inspiring discussions with many colleagues and with William C. Parke, Abigail Salyers, Dorothy Aein, and Raymond Shirven.

We would like to express our appreciation for the generous support from The George Washington University Committee on Research and the National Cancer Institute (NIH research grant CA 04989-BBC).

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*THE NATURE OF THE ENERGY REQUIREMENT FOR AMINO ACID INCORPORATION BY ISOLATED MITOCHONDRIA AND ITS SIGNIFICANCE FOR THYROID HORMONE ACTION**

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Reports from several laboratories[1–4] have now demonstrated that isolated mitochondria will incorporate C^{14}-labeled amino acids into mitochondrial protein without the addition of supernatant fractions. The incorporation is dependent on the energy derived from substrate oxidation, and Truman and Korner[3] have noted that oxidizable substrate provides a better energy source than added ATP. It is the purpose of this paper to report that although this process is energy-dependent, it is largely insensitive to specific inhibitors of ATP formation, and therefore it does not appear to require ATP. This finding has a special significance for the possible control of protein synthesis by the thyroid hormones.

Materials and Methods.—Rat liver mitochondria were prepared by conventional methods[5] and incubated in air at 37°C in a metabolic shaker. The incubation medium is described in the legend of Table 1, and no additions of cell-sap or other supernatant fractions were made. The amino acid mixture which was used had the following molar proportions: alanine 1.0, arginine 0.2, aspartic acid 0.4, asparagine 0.2, cystine 0.2, glutamic acid 0.7, glutamine 0.25, glycine 1.0, histidine 0.1, lysine 0.5, methionine 0.3, phenylalanine 0.25, proline 0.35, threonine 0.4, tyrosine 0.3, and valine 0.5. DL-leucine-1-C^{14} with a specific activity of 4.03 mc/mM was used as the labeled amino acid. Aliquots of the incubation medium containing about 3 mg protein were treated as described by Truman and Korner,[3] and counted until at least 5,000 counts were recorded.

Results and Discussion.—Table 1 shows the rate of leucine-1-C^{14} incorporation under a variety of conditions. For each addition the time course of incorporation was determined, and in most cases the rate was linear up to 1 hr. Table 1 gives these initial linear rates. The data show that leucine-1-C^{14} incorporation was almost completely blocked by both antimycin A and 2,4-dinitrophenol, but it was not inhibited by oligomycin at concentrations which block the terminal phosphorylation steps leading to ATP formation.[6, 7] Higher levels of oligomycin inhibited amino acid incorporation 30–40 per cent, possibly due to a partial inhibition of succinate oxidation.[6] The addition of either ADP or AMP also slowed the rate of incorporation markedly. These findings show that amino acid incorporation in mitochondria

TABLE 1

AMINO ACID INCORPORATION BY ISOLATED RAT LIVER MITOCHONDRIA

Preparation	Addition	Rate of leucine-1-C^{14} incorporation (counts/min/mg protein/hr)	Per cent control
1	Complete system	49.0	—
"	" plus 10 μg/ml antimycin A	0.61	1.2
"	" plus 2×10^{-4} M 2,4-dinitrophenol	2.55	5.2
"	" plus 0.3 μg/ml oligomycin	51.9	106
"	" plus 5 mM ADP	22.9	47
"	" plus 5 mM AMP	5.0	10
"	" 0 time	0.28	0.6
2	Complete system	38.1	—
"	" plus 3.0 μg/ml oligomycin	23.3	61
"	" plus 12.0 μg/ml oligomycin	24.6	65
"	" plus 5×10^{-5} M thyroxine	47.5	125
"	" plus 5×10^{-5} M tetraiodothyroacetic acid	67.7	178
"	" plus 10 μg/ml antimycin A	0.46	1.2
"	" plus 2×10^{-4} M 2,4-dinitrophenol	3.08	8.1
"	" 0 time	0.22	0.6

Incubation conditions: 110 mM sucrose, 10 mM phosphate, 25 mM Tris, pH 7.4, 50 mM KCl, 10 mM nicotinamide, 18 mM DPN^+, 10 mM $MgCl_2$, 10 mM succinate, 1 mg/ml of the amino acid mixture, 0.5 μc/ml DL-leucine-1-C^{14}, 37°C; 3.5 and 3.0 mg mitochondrial protein/ml incubation medium for preparations 1 and 2, respectively.

does not require ATP, although it is dependent on substrate oxidation and energy conservation. This suggests that high energy intermediates in the phosphorylation process may serve as at least a partial energy source for the incorporation process. Similar conclusions have been drawn about the energy requirements for ion accumulation in mitochondria.[8]

Since previous work by this author[9, 10] indicated that thyroxine and certain of its analogues inhibit the terminal steps of the phosphorylation process, the effect of these compounds on amino acid incorporation was also tested. $5 \times 10^{-5}M$ thyroxine, which inhibits ATP-P^{32} exchange by about 40 per cent, stimulated amino acid incorporation by 25 per cent. However, $5 \times 10^{-5}M$ tetraiodothyroacetic acid, which is a potent inhibitor of phosphorylation by blocking the ATP-P^{32} exchange, virtually doubled the rate of leucine-1-C^{14} incorporation. Thus, the thyroid hormones and some of their derivatives may actually stimulate protein synthesis in mitochondria by slowing the formation of ATP and diverting high energy intermediates into the amino acid activation process.

The fact that ATP does not appear to serve as the main energy source for mitochondrial amino acid incorporation makes it unlikely that the currently accepted scheme of amino acid activation[11] is active in these organelles. Since Truman and Korner[12] have shown that mitochondria can supply activated amino acids for incorporation into microsomal protein, the findings reported in this paper may also indicate that ATP need not be the immediate energy source for protein synthesis in the cytoplasm.

Summary.—Amino acid incorporation by isolated liver mitochondria has been found to be an energy-dependent oligomycin-insensitive process. This finding is consistent with the discovery that thyroxine and certain of its analogues increase the rate of amino acid incorporation although they inhibit the terminal steps of the phosphorylation process.

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THYMIDINE-REQUIRING MUTANTS OF PHAGE T4,†*

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Infection with phage T2 can overcome the thymine requirement of a mutant of *Escherichia coli*.[1] Specifically, infection induces a thymidylate synthetase that is physically[2] and enzymatically[3] distinguishable from a normal cell's thymidylate synthetase. The new enzyme appears in cells that have their own enzyme as well as in cells, such as *E. coli* 15T$^-$ or B3, that cannot make their own thymidylate synthetase. The thymidylate synthetases induced by T2 and T6 are different from each other.[3]

To understand the nature of the phage's genetic control over the new enzyme, we have isolated and studied T4 mutants that have lost the ability to grow in a thymineless strain of *E. coli* without exogenous thymidine. The location of the phage mutants on the genetic map has critical implications regarding the timing mechanism for gene expression.

Materials and Methods.—Tris-glucose: NaCl, 5.8 gm; KCl, 3.7 gm; $CaCl_2 \cdot 2H_2O$, 0.15 gm $MgCl_2 \cdot 6H_2O$, 0.10 gm; NH_4Cl, 1.1 gm; $FeCl_3$, 2.7×10^{-4} gm; Na_2SO_4, 0.142 gm; KH_2PO_4, 0.272 gm; 50% glucose (w/v), 10 ml; tris(hydroxymethyl)aminomethane, 12.1 gm; H_2O, to make 1 liter; pH adjusted to 7.4 with 12 *N* HCl.

Tris-glucose-thymidine: Tris-glucose plus 10 μg/ml thymidine.

Glycerophosphate tris-glucose agar plates: These synthetic plates were made with the tris-glucose ingredients with the addition per liter of 0.2 mg thymidine, 10 ml "vitamin-free" casein hydrolysate (acid) (Nutritional Biochemicals Corporation), 0.02 gm L-tryptophan, and 0.10 gm β-glycerophosphoric acid disodium salt (Eastman) replacing the KH_2PO_4. In addition, the bottom agar contained 12.0 gm Difco agar per liter, the top agar 7.0 gm. The plates were poured with approximately 30 ml of bottom agar per plate. The glucose for the bottom agar was autoclaved separately.

Tryptone agar plates: These plates were made with 10 gm Difco tryptone and 5 gm NaCl per liter of H_2O to which was added 12 gm Difco agar for the bottom layer and 7 gm Difco agar for the top layer.

Bacterial strains: *E. coli* B3, a thymine-requiring strain of *E. coli* deficient in thymidylate syn-

thetase activity, was originally isolated by Dr. S. Brenner;[4] *E. coli* BR2 was used for most of the genetic crosses; *E. coli* B and *E. coli* BB were used for growth studies.

Phage strains: T4BO$_1$, osmotic shock-resistant,[5] was the standard phage type from which mutants were isolated. The T4D mutants used for mapping were obtained as follows: *r*47, *r*48, *r*67, *tu*41 from Dr. A. H. Doermann; *am*81, *am*130, *am*134 from Dr. R. H. Epstein; *am*116 from Dr. R. S. Edgar.

Isolation of td mutants: Mutagenic enrichment of phage with thymidineless (*td*) mutants was the key step in the isolation procedure. Phage were treated in 0.2 *M* hydroxylamine and 0.14 *M* sodium phosphate buffer at pH 6.0 for 10 hr at 37°C. The reaction was quenched by diluting 50-fold into broth containing 2% acetone.[6] The surviving fraction was approximately 10^{-3}. To eliminate chemically induced heterozygotes,[7, 8] the treated phage were grown for one step in *E. coli* B, yielding the final mutagenized phage stock. By visual inspection, at least 4% of the phage from the mutagenized stock made plaques that appeared mutant, about 2% being *r* mutants.

The mutagenized stock was plated on tryptone agar with an additional 200 μg thymidine, using *E. coli* B3 as indicator. From these plates an assortment of 200 plaques were picked and suspended in 2 ml of *E. coli* B3. The B3 suspension was made by growing the cells in tris-glucose-thymidine to about 5×10^8/ml, centrifuging and washing two times in tris-glucose, and finally resuspending in tris-glucose at 1×10^9/ml. The phage were incubated without aeration at 37°C for 3 hr and then chloroformed. Any tube that failed to clear was suspected of having been inoculated with *td* mutants. Controls showed that standard T4 phage will cause clearing within two to three hr.

Phage from 15 of the 200 plaques failed to clear the cell suspension. Of the 15, the 7 making the largest plaques were screened further by comparing their burst sizes in *E. coli* B3 in tris-glucose-thymidine and tris-glucose. Phage from two plaques, labeled *td*8 and *td*15, showed about a 20-fold lower burst of phage when thymidine was omitted. Detailed growth studies are described in a later section. The significant reduction in burst size when thymidine is omitted from the B3 culture is the property that defines a *td* mutant.

To help eliminate other mutations that might have accumulated during the hydroxylamine treatment, *td*8 was backcrossed five times with the original T4BO$_1$, and *td*15 was backcrossed once. All studies were done with these backcrossed strains.

Plaque morphology and simple tests for td mutants: Plaques of *td* mutants are indistinguishable from *td*$^+$ on tryptone agar; but the mutants can be distinguished on tris-glucose plates. The procedure is to preadsorb the phage for 15 min to B3 that was grown to about 5×10^8/ml in tris-glucose-thymidine. The infected cells are then plated on tris-glucose plates using 2 ml top agar containing 4 μg/ml thymidine and 2 drops of a culture of B3 grown overnight in tris-glucose-thymidine. The *td* mutants show appreciably smaller plaques than *td*$^+$ any time after 8 hr incubation at 37°C. The plaques are shown in Figure 1*a*. Without preadsorption the efficiency of plating of *td* phage is reduced.

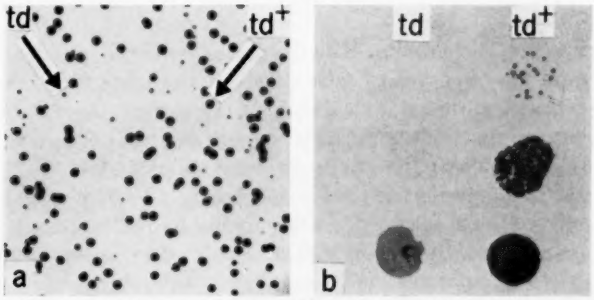

FIG. 1.—(*a*) Comparative morphology of *td* and *td*$^+$ plaques on glycerophosphate tris-glucose agar plates with B3 indicator, photographed after 14 hr incubation at 37°C.

(*b*) Spots of *td* and *td*$^+$ phage on glycerophosphate tris-glucose agar plates with B3 indicator photographed after 8 hr incubation at 37°C. The number of phages deposited in each spot varies from about 20 in the top row to about 10^4 in the bottom row.

A simple spot test was developed that distinguishes the *td* mutants from td^+. Using the same type of synthetic plates but now seeded with 5 drops of B3 in the overlay agar, roughly 0.002 ml of phage are deposited on the hardened overlay agar with a melting point capillary tube. The results are shown in Figure 1*b*. The test is not reliable when more than 10^4 phage are deposited in a spot.

The difference in plaque morphology between td^+ and *td* has been used to simplify subsequent isolations of *td* mutants. The mutagenized phage stock is plated on tris-glucose agar seeded with *E. coli* B3. The distinctly smaller plaques are picked and the phage tested for plaque size on two kinds of plates: tris-glucose agar seeded with B3 and tryptone agar seeded with B. We select phage which, compared with td^+, give standard-size plaques on B and small plaques on B3. Two mutants fitting this description were chosen and both gave a 20-fold lower burst size in B3 when grown in tris-glucose, compared with growth in tris-glucose-thymidine. These mutants were labeled *td*9 and *td*10.

D. L. Wulff and K. Metzger (*Virology*, 1963, in press) have independently isolated a thymine-requiring mutant of phage T4.

TABLE 1

BURST SIZE OF T4 MUTANTS IN THYMIDINE-DEPENDENT HOST *E. coli* B3 AND THYMIDINE-INDEPENDENT HOSTS *E. coli* B AND BB

	Phage Yield/Infected Cell			
	B3		B	BB
Phage	10 μg/ml thymidine	No thymidine	No thymidine	No thymidine
td^+	140	95	58	43
*td*8	100	7	60	46
*td*9	72	4	52	40
*td*10	120	6	42	41
*td*15	150	8	...	...

The cells were grown to 5×10^8/ml in tris-glucose-thymidine, washed two times in tris-glucose by centrifuging and finally resuspended at a concentration of 10^8/ml in tris-glucose supplemented with 0.002 *M* KCN and 20 μg/ml L-tryptophan. 0.1 to 0.2 phage per bacterium were added, allowed to adsorb for 6 min at 37°C without aeration, and then diluted 10^4-fold into tris-glucose with thymidine added as shown in the table. The cells were incubated at 37°C with gentle aeration for 100 min, lysed with chloroform, and assayed.

Results.—Evidence that td mutations affect a genetic region controlling the appearance of thymidylate synthetase activity: Table 1 shows the growth of *td* mutants in the presence and absence of added thymidine. *E. coli* B3 grown at 37°C requires thymidine for growth in tris-glucose because it has a very low level of thymidylate synthetase activity.[4] Since *td* mutants can grow normally on B and BB, and cannot grow well in B3 without added thymidine, we conclude that the mutations affect a phage genetic region that controls the appearance of thymidylate synthetase activity.

One-step growth: The pattern of early phage synthesis in *td*8-infected B3 is shown in Figure 2*a*. Synthesis begins at about the same time whether or not thymidine is added, but the rate is less without thymidine. Results from the same experiment, but at later times after infection, are shown in Figure 2*b*. The addition of thymidine increases the phage yield by about 20-fold. The addition of FUDR is known to inhibit thymidylate synthetase.[3] FUDR plus uridine (Fig. 2*b*) consistently reduced the phage yield. Uridine alone had little or no effect. Similar results have been obtained with *td*9, *td*10, and *td*15. In no case does FUDR completely eliminate phage production.

It might be expected that the loss of a thymidine-synthesizing system would be a handicap for the phage even in the thymidine-independent host. Yet *td* phages seem to grow more or less normally on B or BB and make plaques which are indistinguishable from those of wild type on these hosts. Possibly a disadvantage would be clearly apparent if phage yields were high. This possibility was

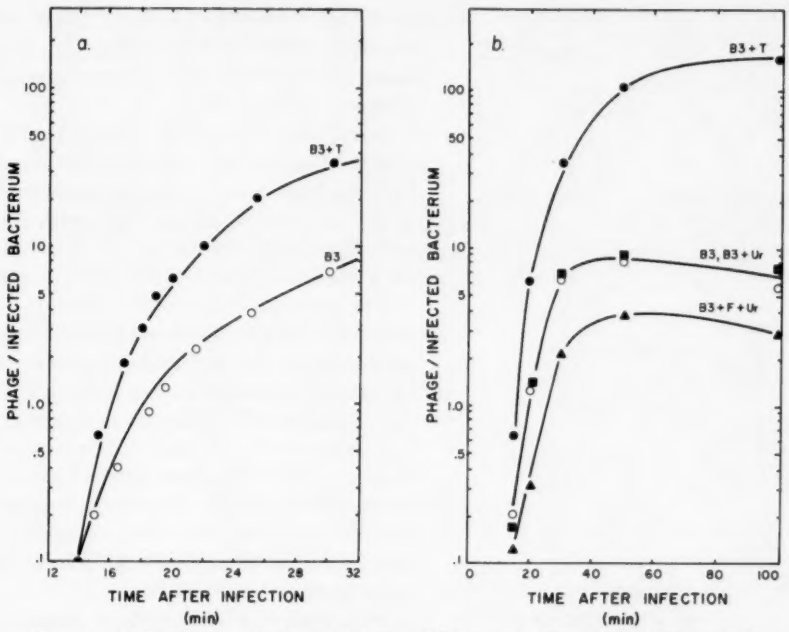

FIG. 2.—(*a*) Production of phage *td*8 in *E. coli* B3 at early times after infection in the presence and absence of thymidine. The conditions of infection are described in the legend to Table 1. When samples were taken, 1 ml of the culture was added to 1 ml nutrient broth, and lysed with chloroform. The growth media are indicated on the drawing. B3 = control containing tris-glucose; T = 10 μg/ml thymidine.

(*b*) Same experiment as in 2*a*, showing the effect of various additives over the entire course of the infection. The growth media are indicated on the drawing. B3 = control containing tris-glucose; T = 10 μg/ml thymidine; F = 5 μg/ml fluorodeoxyuridine; Ur = 20 μg/ml uridine.

tested by infecting BB with td^+ and *td*8 and inhibiting lysis by superinfecting at later times[9] as described in Figure 3. Even though the burst sizes exceeded 500 phage per bacterium there was no significant difference between *td*8- and td^+-infected cells, and addition of thymidine had no noticeable effect.

Complementation: Complementation was measured by the growth of phage in mixedly infected B3, without thymidine. Table 2 shows the burst sizes from B3 infected with all possible pairs of mutants. The first row of numbers shows that the effect of the td^+ gene together with any of the *td* mutants is to allow a normal burst, the burst containing roughly equal numbers of parental types. But growth of any pair of mutants was not measurably better than the growth of either mutant alone. Therefore, the four *td* mutants isolated show no complementation as measured by the burst size. We use the lack of complementation in the *trans* configuration as the criterion for tentatively placing the four mutants in the same cistron.

Genetic mapping of the td mutants: Each *td* mutant recombines with the other three, the frequencies ranging from 1 to 4 per cent.

To map the *td* mutants on the circular map of T4,[10] *td*8 which had been backcrossed five times to $T4BO_1$ and then backcrossed four times to T4D was used.

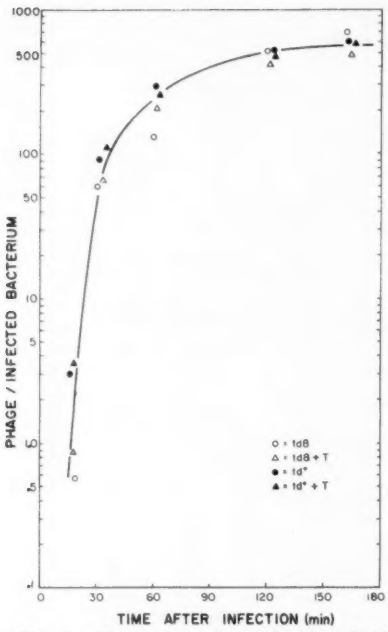

FIG. 3.—Comparison of growth rates of td^+ and *td*8 under conditions of lysis inhibition. *E. coli* BB was grown to 5×10^8/ml in tris-glucose, and supplemented with tryptophan immediately before infection. The bacteria were infected with either td^+ or *td*8 at a multiplicity of 3–5 phage per bacterium and superinfected at times 13, 32, 62, and 122 min after infection with seven phage of the same type per bacterium. Indicated cultures were supplemented with 10 μg/ml thymidine. Cells were diluted 100-fold into broth at the given times and lysed with chloroform before assaying.

Four-factor crosses (Table 3) place *td*8 between *am*134 and *r*67 (Fig. 4), well removed from the so-called "early region," between *r*47 and *r*48.[11]

Discussion.—*E. coli* B3 lacks thymidylate synthetase activity when grown at 37°C.[4] From the comparative growth of *td* mutants on B, BB, and B3, with and without added thymidine, we conclude that the mutants are deficient in the ability to produce thymidylate synthetase activity. This has been confirmed by examination of the enzymatic activities in extracts from cells infected with each of the *td* mutants.[12] Thus, there is now direct evidence that T4 asserts genetic control over the production of thymidylate synthetase activity. The lack of complementation among the four *td* mutants suggests that these mutants affect the same protein.

B3 infected with *td* phage produces about 8 phage per bacterium in the absence of added thymidine, about 120 in its presence. Since about 50 phage DNA equivalents of thymidine are available via breakdown of bacterial DNA,[13] why is so little phage produced in the absence of added thymidine? An explanation is suggested by experiments which show that phage-precursor molecules mature after FUDR is added to a T4-infected culture, but that 20–30 phage-precursor molecules remain unmatured whatever the size of the pre-existing pool.[14] Hence, a minimum pool of this size may be necessary before there can be any phage maturation. Since only 50 per cent of the bac-

TABLE 2

COMPLEMENTATION OF *td*-MUTANTS

	td^+	*td*8	*td*9	*td*10	*td*15
td^+	70	60	120	90	120
*td*8		3	7	6	5
*td*9			4	5	9
*td*10				4	6
*td*15					5

A number within the matrix is the burst size of phage from B3 cells mixedly infected with the two phage types heading the corresponding row and column.

E. coli B3 was grown to 1 to 2×10^8/ml in tris-glucose-thymidine, washed two times by centrifuging, and concentrated to 5×10^8/ml, all in tris-glucose. Twenty μg/ml L-tryptophan was added for adsorption. One ml aliquots of the concentrated B3 were infected with the indicated pairs of phages, each phage at a multiplicity of infection of 4. The mixtures were aerated at 37°C for one hr, lysed with chloroform, and assayed. In the first row the numbers comprise roughly equal amounts of each parental type.

TABLE 3

RELATIVE NUMBERS OF *td* AND *td*$^+$ AMONG RECOMBINANTS IN FOUR-FACTOR CROSSES

Recombinants	*r*67*tu*41*td*8 × *am*134 Exp. 1	Exp. 2	Recombinants	*tu*41*td*8 × *r*67*am*134 Exp. 1	Exp. 2
*r*67*am*$^+$*tu*$^+$*td*	22	67	*r*$^+$*am*$^+$*tu*$^+$*td*	18	33
*r*67*am*$^+$*tu*$^+$*td*$^+$	1	12	*r*$^+$*am*$^+$*tu*$^+$*td*$^+$	1	0
r$^+$*am*$^+$*tu*41*td*	18	45	*r*67*am*$^+$*tu*41*td*	14	26
r$^+$*am*$^+$*tu*41*td*$^+$	5	29	*r*67*am*$^+$*tu*41*td*$^+$	7	8

Recombinants	*r*47*r*48*td*8 × *am*116	*r*47*r*48*td*8 × *am*81	*r*47*r*48*td*8 × *am*130
*r*47*am*$^+$*r*$^+$*td*	20	11	16
*r*47*am*$^+$*r*$^+$*td*$^+$	20	12	13
r$^+$*am*$^+$*r*48*td*	17	26	21
r$^+$*am*$^+$*r*48*td*$^+$	32	51	61

terial DNA appears in phage,[13] the low yield is not surprising. The partial sensitivity of the yield to FUDR shows that thymidylate synthetase activity is indeed the factor limiting burst size.

Why does the phage possess the *td*$^+$ function? Flaks and Cohen[15] have suggested the reasonable explanation that a large increase in thymidylate synthetase activity is needed after infection to match the increased need for thymine. They suggested that thymidylate synthetase activity is a rate-limiting step in phage synthesis. We find no support for this explanation. In tris-glucose, *td* mutants grow approximately as well as *td*$^+$ in *E. coli* B and BB, even under conditions of lysis inhibition where the burst size is as high as 500. Furthermore, DNA synthesis starts at the same time and proceeds at the same rate in cultures of BB infected with *td*8 or *td*$^+$.[16] Only under the conditions of extreme thymidine deprivation present in *E. coli* B3 do we find a significant distinction between *td* and *td*$^+$. The *td*$^+$ genetic region might normally confer only a slight advantage to the phage. Possibly its true worth cannot be fully appreciated outside such environments as colons and sewers.

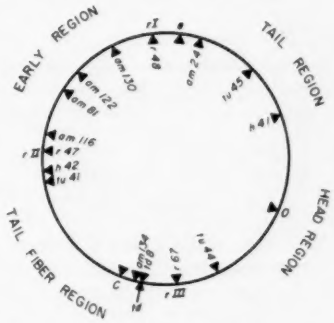

FIG. 4.—The location of the *td* locus on the genetic map of T4.[17]

The position of *td* on the T4 genetic map is relevant to theories of phage development. In a study of the physiology of T4 mutants, Edgar, Denhardt, and Epstein[17] have observed that phage mutants affecting functions which are expressed at about the same time are arranged in contiguous regions of the map. For example, the genetic region between *r*II and *r*I seems to contain only genes responsible for "early functions," controlling the formation of enzymes that appear early in phage development before DNA synthesis begins. In cells infected with T-even phages, thymidylate synthetase activity starts to increase approximately five minutes after infection.[15, 18, 19] In T6, dCMP hydroxymethylase activity appears immediately after infection, and lysozyme activity ten minutes after infection.[18] Therefore, *td* should fall in the "early region," possibly between *am*122, which seems to control dCMP hydroxymethylase,[19] and *e*,[20] which controls the phage lysozyme. Contrary to these expectations, the *td* locus is nowhere near this region,

but is well within a "late region" containing cistrons that control the formation and assembly of phage coat protein. Therefore, phage mutants affecting functions which are expressed at about the same time need not be arranged in contiguous regions of the map.

Summary.—Four mutants (*td*) of phage T4 have been isolated. They are deficient in their ability to grow in a thymine-requiring strain of *E. coli* without exogenous thymidine, but grow normally when thymidine is added. The mutants are all in the same cistron, which is responsible for the appearance of phage-controlled thymidylate synthetase activity. The *td* mutants map in a position far removed from a region controlling other functions that are also expressed early in phage development, showing that phage mutants affecting functions which are expressed at about the same time need not be arranged in contiguous regions of the map.

Note added in proof: Recent experiments have shown that thymine, as well as thymidine, is effective in supporting growth of *td* mutants in *E. coli* B3. Therefore, the *td* mutants are, strictly speaking, thymine-requiring.

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† The following abbreviations are used: FUDR, 5-fluorodeoxyuridine; dCMP, deoxycytidine-monophosphate.

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*N-ACETYLAMINO ACIDS AND PROTEIN SYNTHESIS**

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The ordinarily free amino groups of N-terminal residues in polypeptide chains are blocked in some instances by acetyl groups, e.g., in melanocyte-stimulating hormone (α-MSH),[1] and in the following proteins: horse heart cytochrome c,[2] hemoglobin F_1,[3] egg albumin,[4] histones,[5] turnip yellow mosaic virus (TYMV),[6] and tobacco mosaic virus (TMV) proteins.[7]

The question arises whether the introduction of these acetyl groups occurs at an early, intermediary, or late stage of protein synthesis. Some time ago we described the conversion of phenylalanine and leucine into their N-acetyl derivatives by rat liver slices,[8] reactions which have remained without a known metabolic function. The existence of N-acetylproteins has now led us to consider the possibility that the acetylation of amino acids serves the purpose of furnishing the N-terminal acetyl groups of N-acetylproteins. As the first step in exploring the role of acetylation in protein synthesis, we have investigated the reactivity of N-acetylamino acids in amino acid activating systems and the transfer of the products to sRNA. We now report results demonstrating that several N-acetylamino acids, notably N-acetyltyrosine, enter the early reactions of protein synthesis as measured by the formation of hydroxamates, by the promotion of the inorganic pyrophosphate-ATP exchange, and by incorporation into sRNA. The acetyl substituent is not removed from the amino acid during these transformations.

Materials.—Fresh calf liver was obtained from a local slaughterhouse and was stored at $-20°$. Disodium ATP, N-acetyl-L-alanine, and N-acetyl-L-glutamic acid were obtained from Sigma Chemical Company; L-tryptophan, L-glutamic acid, L-tyrosine, L-histidine, N-acetyl-L-tryptophan, N-acetyl-L-tyrosine, N-acetyl-L-histidine, and N-acetyl-L-tyrosine ethyl ester from California Corporation for Biochemical Research; L-alanine and rat liver soluble RNA from the Nutritional Biochemical Company; P^{32}-pyrophosphate with a specific activity of 6.1 mc/mmole from Nuclear-Chicago Co.; and tritiated acetic anhydride with a specific activity of 0.49 mc per mg from New England Nuclear Corp. C^{14}-L-Leucine, 7.64 μc per mg, was obtained from Schwarz BioResearch, Inc.

Methods.—Preparation of enzyme: Fresh or frozen liver was homogenized at top speed in a Waring blendor for 1 min in two volumes of either medium X of Zamecnik and Keller[9] with 0.01 *M* Tris buffer instead of phosphate buffer, or in the medium of Matthaei and Nirenberg.[10] The homogenate was centrifuged at 30,000 $\times$ *g* for 10 min and then at 105,000 $\times$ *g* for 1 hr. The supernatant fraction was filtered through glass wool and dialyzed against 10 volumes of homogenizing medium for 6–12 hr. All operations were carried out at 4°. The protein concentration in the dialyzed enzyme preparation was 25 mg per ml.[11, 12]

RNA was prepared from baker's yeast by phenol extraction according to Holley *et al.*,[13] and from calf liver by alcohol precipitation as described by Berg *et al.*[14] In both procedures, RNA was dissolved in 1 *M* NaCl prior to final reprecipitation.

The nucleic acid content of the crude homogenate determined by the method of Warburg and Christian[12] was 0.75 mg per ml. DNA and RNA were determined with diphenylamine and orcinol reagents, respectively;[15] 70% of the total nucleic acid was RNA.

Salt-free hydroxylamine was prepared by the method of Beinert *et al.*[16] Synthetic N-acetyl-L-tyrosine hydroxamate was prepared from N-acetyl-L-tyrosine ethyl ester by the method of Anderson.[17] N-acetyl-L-tyrosine labeled with tritium in the acetyl group (N-acetyl-(H^3)-tyrosine) was prepared by adapting the method of du Vigneaud and Meyer to a microscale.[18] After dilu-

tion with carrier N-acetyl-L-tyrosine, the product had a specific activity of 1.5×10^6 cpm per μmole. More than 90% of the counts coincided with authentic N-acetyl-L-tyrosine when the labeled compound was chromatographed on Whatman No. 1 paper in *n*-butanol saturated with 0.1 *N* HCl. A single spot with R_f 0.7 corresponding to the R_f of authentic N-acetyl-L-tyrosine was observed on spraying with 0.44% aqueous potassium ferricyanide followed by alcoholic ferric chloride reagent (5 gm $FeCl_3$, 5 ml concentrated HCl, and 95 ml 95% ethanol). The acetylamino acid was not contaminated by free tyrosine (R_f 0.3 in this system) and no ninhydrin-positive spot was detected. Thin layer chromatography on silica gel G (according to Stahl) in *n*-butanol:acetic acid:water, 80:20:20, gave results confirming the identity of the labeled acetyltyrosine.

Hydroxamate formation was assayed by the method of Conway *et al.*[19] The products from the hydroxamate assay were treated according to Hoagland *et al.*,[20] and analyzed by descending chromatography on Whatman No. 1 paper in *n*-butanol saturated with 0.1 *N* HCl. The hydroxamate spots were visualized by spraying with alcoholic $FeCl_3$ reagent. Radioactivity was determined by counting 1 cm lengths of the paper in toluene scintillator before spraying.[21]

The amino acid-promoted ATP-P^{32}-inorganic pyrophosphate exchange was assayed by the method of Hoagland.[22] For radioactivity determinations, 0.1 ml aliquots of aqueous solutions were added to "polyether" solution (containing 750 ml 1,4-dioxane, 125 ml anisole, 125 ml bis (2-methoxyethyl) ether, 4 gm 2,5-diphenyloxazole and 100 mg 1,4-bis-2-(5-phenyloxazolyl)-benzene) and counted in a Packard liquid scintillation counter.

Acceptor activity of soluble RNA for N-acetyl-L-tyrosine was measured according to Berg *et al.*[14] The ethanol-salt precipitates of RNA were transferred directly into liquid scintillation vials with polyether scintillator solution, or the precipitates were filtered onto Whatman 3MM filter paper disks (2.3 cm diameter) washed twice with ethanol-salt, and the paper counted in toluene scintillator.

Release of N-acetyl tyrosine from sRNA: N-acetyl-(H^3)-tyrosyl sRNA was prepared by the procedure of Berg *et al.*[14] for aminoacyl sRNA, and the acetylamino acid removed from the RNA by incubation in 1 *M* carbonate-bicarbonate buffer, pH 10, at 37° for 1 hr. RNA was precipitated with alcohol, removed by centrifugation, and the supernatant taken to dryness. The residue was acidified, extracted with acetone, and the extract chromatographed on paper as described above for free acetyltyrosine.

Results and Discussion.—Hydroxamate formation: Carboxyl activation as measured by hydroxamate formation occurs on incubation of several N-acetylamino acids with the amino acid activating system from calf liver (Table 1). The most

TABLE 1

HYDROXAMATE FORMATION

Amino acid	Units of activity per 1 mg protein: Free amino acid	N-acetylamino acid
Tryptophan	0.31; 0.59*	0.181
Alanine	0.059	0.014
Glutamic acid	0.064	0.058
Tyrosine	0.10	0.23; 0.13;* 0.14*
Histidine	0.005	0.068

* Enzyme stored at −20° for 2 weeks.

Frozen enzyme was stored at −20° for one week, except as indicated. Assay tubes contained in μmoles: Tris-chloride buffer pH 7.6, 100; $MgCl_2$, 8; NH_2OH, 1,000; ATP, 10; amino acid or N-acetylamino acid, 40; and 0.4 ml enzyme, in a total volume of 1.1 ml. Tubes were incubated for 1 hr at 37°, and the reaction was stopped with 3 ml of a solution containing 10% $FeCl_3$ and 5% TCA in 0.67 *N* HCl. Optical density at 540 mμ was measured, and an O.D. value of 0.16 was taken to correspond to 0.25 μmoles hydroxamate per ml. One unit of enzyme catalyzes the formation of 1 μmole hydroxamate per hr. Values obtained in the absence of amino acids have been subtracted.

active compound was N-acetyltyrosine, but a positive reaction was also obtained with the N-acetyl derivatives of tryptophan, alanine, glutamic acid, and histidine. Other N-acetylamino acids remain to be tested. Since N-acetyltyrosine showed the highest activity in the hydroxamate assay, all further experiments were carried out with this amino acid. The quantity of hydroxamate formed was the same as, or somewhat greater than, with free tyrosine, and these activities in turn were com-

parable to the values reported in the literature for free amino acids. For example, a value of 0.012 units per mg has been reported for L-tyrosine hydroxamate formation in crude extracts of hog pancreas,[23] and a value of 0.116 units per mg for phenylalanine in a crude *E. coli* extract.[19] Under the same assay conditions, no hydroxamate-positive material was formed with acetate as the substrate. That the activation product is a derivative of N-acetyltyrosine has also been shown with N-acetyl-(H^3)-tyrosine. Paper chromatography of authentic N-acetyl-L-tyrosine hydroxamate gave two spots,[24] one with an R_f of 0.6–0.7 appearing immediately on spraying, and another with R_f 0.2 appearing 20–30 hr after spraying. On chromatography of the hydroxamate formed from labeled acetyltyrosine, 70 per cent of the counts applied to the paper migrated with R_f 0.2. No counts were present in the region of R_f 0.7. The radioactive spot was ninhydrin-negative and was not identical with acetylhydroxamate.

Pyrophosphate-ATP exchange: N-acetyltyrosine was active in the amino acid-promoted PP^{32}-ATP exchange, and the extent of exchange was comparable in magnitude to the exchange obtained in the presence of free tyrosine (Table 2).

TABLE 2

AMINO ACID-PROMOTED ATP-PP^{32} EXCHANGE

Amino acid	mμmoles of PP^{32} exchanged per mg of protein
Control, boiled enzyme	0
N-acetyl-L-tyrosine	8; 9
L-tyrosine	17
L-tryptophan	0; 1
N-acetyl-L-tryptophan	1
Sodium acetate	0

Assay tubes contained in μmoles: Tris-chloride buffer pH 7.6, 100; $MgCl_2$, 4; KF, 60; ATP, 5; PP^{32}, 4 (containing approximately 550,000 cpm); amino acid or N-acetylamino acid, 4; and 0.4 ml enzyme in a total volume of 1 ml. Tubes were incubated at 37° for 10 min. The reaction was stopped with TCA; and ATP and PP^{32} were separated by charcoal adsorption. The values for exchange occurring in the absence of added amino acid have been subtracted.

TABLE 3

INCORPORATION OF N-ACETYL-(H^3)-TYROSINE INTO sRNA

	N-acetyl-(H^3)-tyrosine cpm in sRNA	C^{14}-L-leucine cpm in sRNA
Control, no enzyme	2,580	610
Complete	(*a*) 9,640	(*a*) 1,490
	(*b*) 6,480	(*b*) 1,550
Per cent incorporation, average of (*a*) and (*b*)	0.9	0.5

Assay tubes contained in μmoles: Tris-chloride buffer, pH 7.4, 50; $MgCl_2$, 4; KCl, 5; ATP, 5; glutathione, 2; N-acetyltyrosine, 600,000 cpm., or C^{14}-L-leucine 188,000 cpm, 0.4; sRNA nucleotide, 1; 100 μg crystalline bovine serum albumin; and 0.05 ml enzyme in a total volume of 0.5 ml. Incubation at 30° for 10 min. In some experiments, 1.5 mg carrier yeast sRNA was added after stopping the reaction with ethanol-salt. This addition had little effect on recovery of radioactivity.

These values lie in the same range as those reported in various laboratories for free amino acids.[25–27]

Transfer to sRNA: The standard amino acid activating system was incubated (in the absence of microsomes) with N-acetyl-(H^3)-tyrosine. The aminoacyl sRNA fraction isolated by the procedure described above was radioactive and contained 0.9 per cent of the radioactivity added to the incubation medium. Under the same conditions, incorporation into sRNA from C^{14}-leucine was 0.5 per cent. On exposure of the labeled sRNA to pH 10, about 25 per cent of the H^3-label was liberated in a form which paper chromatography showed to be identical with free

N-acetyltyrosine, R_f 0.7. The acetyl group therefore remains attached to the amino acid during activation and transfer to sRNA.

Discussion.—As shown by the present results, certain N-acetylamino acids undergo the reactions which are generally believed to initiate protein synthesis from free amino acids. The results also demonstrate that the NH_2-group of the amino acid need not be free for carboxyl-activations and transfer to sRNA. However, the nature of the blocking group seems to be of some importance since N-carbobenzoxy-amino acids and certain peptides are apparently unreactive in the amino acid activating system.[25, 26]

The acetylated N-terminal amino acid residues so far identified in proteins are glycine in horse heart cytochrome c,[2] serine in TMV protein[7] and in α-MSH,[1] and methionine in TYMV protein.[6] N-acetyltyrosine, or any of the amino acids listed in Table 1, has not so far been shown to occupy N-terminal positions, but in several proteins known to terminate with an N-acetyl grouping, e.g., the γ-chain of hemoglobin F_1,[3] egg albumin,[4] and histone,[5] the terminal amino acid has not yet been identified. Moreover, in a number of proteins free N-terminal amino groups cannot be detected, indicating perhaps that in these cases also the amino-terminal grouping is masked by substitution.

The utilization of N-acetylamino acids in the early steps of protein synthesis raises the possibility that the acetylation reaction leading to acetylproteins occurs with the free amino acid and not at the sRNA stage or with the completed protein. The purpose of the acetylation reaction is entirely a matter of speculation. In α-MSH the N-acetyl group of the terminal serine residue does not appear to be essential for melanocyte-stimulating activity.[28] Whether and to what extent the acetyl group modifies the properties of acetyl-containing proteins is not known. Although the number of recognized N-acetylproteins is so far relatively small, we would like to suggest a more general role for acetylamino acids in protein synthesis. The evidence presented by Dintzis[29] suggests that peptide chains are begun and grow from the N-terminal end. It would be an obvious advantage to start this process with an N-acetylamino acid or a similarly protected amino acid leaving only the carboxyl function available for peptide bond formation. Selection of an N-acetylamino acid as the chain-initiating unit will automatically fix the N-terminal position of the polypeptide by allowing the chain to grow in one direction only. In order to be generally applicable, the proposed mechanism would have to include the postulate that, in those proteins in which the N-terminal NH_2-group is free, the acetyl group is removed after the polypeptide chain has been completed.

Summary.—N-acetyltyrosine incubated with calf liver extracts undergoes carboxyl-activation as measured by hydroxamate formation and ATP-PP^{32} exchange. Under the same conditions, N-acetyltyrosine can be transferred to sRNA.

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HOMOLOGY BETWEEN BACTERIOPHAGE λ DNA AND E. COLI DNA

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Detailed homology in nucleotide sequences between two DNAs originating from different organisms may be demonstrated by the formation of mixed duplex molecules.[1, 2] While no evidence has been obtained indicating genetic homology, or common nucleotide sequences, between the T series of virulent bacteriophage and the host bacterium *E. coli*,[1] the transduction of host genetic markers with lysogenic bacteriophage is common, suggesting that some homology between the virus and host DNA might exist.

Upon infection of a suitable strain of *E. coli*, the chromosome of the temperate bacteriophage λ enters into a prophage state in which it appears to be attached to a specific site on the bacterial chromosome. It is then replicated at the same rate as the host genome and, in fact, coincidentally with that particular region to which it is presumed to be attached.[3]

The available evidence suggests an addition to the host chromosome rather than substitution of the prophage for an homologous segment of the bacterial genome. In rare cases, however, part of the chromosome of the λ prophage is lost and is re-

placed by a segment of the bacterial genome.[4] This substituting material represents the genetic determinants controlling the utilization of galactose, and it is to this region that the prophage is closely linked. The amount of the displaced λ chromosome is of the order of 25–30 per cent, although this and the quantity of bacterial material replacing it are both somewhat variable.[5]

By the use of the DNA-agar technique it will be shown below that an appreciable segment of the λ phage genome is related to that of the host and other bacterial species and that this homology is characteristic of only a very limited section of the *E. coli* chromosome.

Materials and Methods.—*Escherichia coli* strains B, K12 (λ), and K12 (λ-sensitive) were grown in glucose-minimal salts medium. Except where noted below, all other bacterial cultures were grown in nutrient broth containing glucose.

Bacterial DNA was prepared by the procedure of Marmur[6] and was fragmented by passing it through a French pressure cell at 10,000 psi at a DNA concentration of approximately 1 mg/ml in 2 × SSC (SSC is 0.15 *M* NaCl, 0.015 *M* Na citrate). Molecular weight of the fragments is about 6×10^5 (as measured by Dr. E. Burgi). Fragmented or native DNA was denatured by heating for five min in boiling water in 0.1 × SSC and rapid cooling.

Denatured DNA was trapped in agar by the method of Bolton and McCarthy.[2, 7] Duplex formation was also carried out by the procedures employed by the above authors.

The quantity of DNA remaining trapped in the 3% agar after 2 × SSC washes was determined by dissolving 0.1 gm of the DNA-agar in 0.9 ml of 5 *M* $NaClO_4$ solution and heating at 60°C until all the agar was dissolved. The UV absorption spectrum was then obtained, using a blank containing agar without trapped DNA.

Labeled λ DNA was prepared by UV irradiation of 400 ml of K12 (λ) cells ($\sim 5 \times 10^8$ cells/ml) removed from a culture growing exponentially in nutrient broth-glucose medium. These cells were first washed by centrifugation in tris-glucose medium and then resuspended in 100 ml of tris-glucose medium and irradiated 1 min. Following irradiation the cells were added to 400 ml of tris-glucose medium containing 30 ml nutrient broth and aerated at 37°C. Ten min after irradiation 2 mc P^{32} was added to the culture. Lysis occurs 80 min following the UV irradiation. Upon completion of lysis the bacterial debris was removed by centrifugation for 5 min at 8,000 rpm in a superspeed Servall centrifuge. DNAase was added to the supernatant to remove bacterial DNA. This lysate was then centrifuged for 120 min at 21,000 rpm in a type 30 rotor, Beckman Model L ultracentrifuge. The supernatants were decanted, pellets rinsed with 2 × SSC, and then collected and resuspended in 20 ml of 2 × SSC. This resuspension was finally centrifuged for 5 min at 8,000 rpm to eliminate any traces of bacterial debris. DNA was then extracted from the supernatant by the procedure described by Grossman *et al.*[8]

Results.—*Reaction of λ DNA with E. coli DNA:* Reaction of heat-denatured λ DNA with *E. coli* DNA trapped in agar occurs if the λ DNA has been fragmented to allow it to penetrate the agar gel.[11] Optimal interaction with the trapped DNA results when the amount of fragmented DNA is small compared to that of the bound DNA, thereby limiting a competing reaction in which renaturation takes place between two of the fragmented molecules. This is particularly important in the present studies, since the relatively homogenous bacteriophage DNA fragments

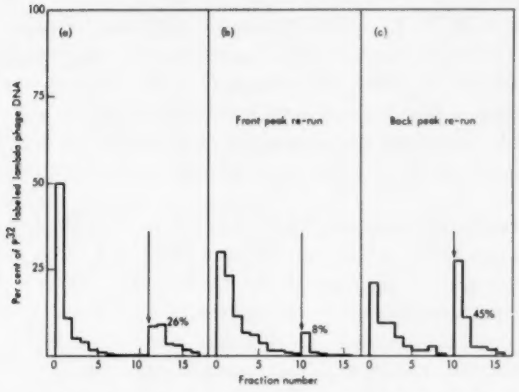

FIG. 1.—Reaction of λ DNA fragments with *E. coli* DNA trapped in agar. After incubation at 60°C ten washes were given at 60°C with 2 × SSC. The temperature was then raised to 75°C, and seven 10 ml fractions of 0.01 × SSC were passed through. Equal aliquots of the λ DNA appearing in the two peaks were each reincubated with a similar agar preparation.

readily renature and the number of homologous sites in the bacterial DNA are very limited.

Accordingly, a small quantity (0.12 μg) of high specific radioactivity λ DNA fragments was incubated at 60°C for 15 hr with 0.5 gm of agar gel containing 150 μg of *E. coli* B DNA. After separation of the bound and unbound portions it was apparent that 26 per cent of the λ DNA fragments had formed a stable duplex structure with the *E. coli* DNA (Fig. 1*a*). This can be shown to be a specific portion of the total λ DNA by reincubation of the two separated portions. The initially unbound portion of λ DNA binds very poorly in a second incubation (Fig. 1*b*) even though it is heat-denatured again before adding to the *E. coli* DNA-agar. On the other hand, that fraction of the λ DNA selected on the basis of its homology with bacterial DNA is seen to be effectively readsorbed (Fig. 1*c*).

In earlier experiments with the DNA-agar technique it was shown that the selection of molecules complementary to the trapped DNA was about 80 per cent efficient.[11] This results mainly from the fact that some 10 per cent or more of the trapped DNA leaks out of the agar during the long incubation period. The best interpretation of the present experiment is therefore that somewhat more than 26 per cent of the λ DNA fragments are homologous to *E. coli* DNA and that the 8 per cent obtained in Figure 1*c* represents the remaining homologous λ DNA. With this correction the best estimate of the fraction of λ DNA homologous to *E. coli* would be about 33 per cent. The failure to obtain 80 per cent efficiency in the readsorption of the originally bound material can be attributed to the presence of *E. coli* DNA leached from the agar when the temperature was raised and the salt concentration reduced, and to the greater tendency of the λ DNA fragments to renature.

Maximal binding of λ DNA fragments to E. coli DNA: The demonstration that a portion of λ DNA may interact with regions of the *E. coli* DNA raises the question

of how much of the host DNA is related to that of the lysogenic phage. The extent of this relationship can be determined by measuring the maximum quantity of λ DNA fragments which can form duplexes with a known amount of denatured *E. coli* DNA in agar. The results of such a determination are shown in Figure 2. As the quantity of λ DNA is raised, the amount of labeled DNA bound to the DNA-agar increases, approaching, as a limit, an amount equivalent to 0.2 per cent of the *E. coli* DNA.

In similar saturation experiments where labeled *E. coli* DNA fragments were incubated with *E. coli* DNA in agar, an amount of labeled DNA equivalent to 80 per cent of that present in the agar could be bound.[11] It is evident therefore that the λ DNA preparation cannot be contaminated with *E. coli* DNA. Moreover, essentially all of the DNA present in the agar can be induced to form duplex structures if homologous nucleic acid fragments are provided.[11] Thus, the interpretation can be made that λ DNA is homologous to only a small part of the host DNA, a portion which is quantitatively assessed by the saturation experiment.

A knowledge of the fractions of the *E. coli* and the λ DNA which are similar enough to form mixed duplex molecules allows a calculation of the ratio of the length of the two molecules. Since the total molecular weight of λ DNA is known,[9] that of *E. coli* may then be calculated:

$$\frac{3.1 \times 10^7 \text{ (molecular weight λ DNA)}}{\text{X(molecular weight } E.\ coli \text{ DNA)}} = \frac{\text{Fraction of } E.\ coli \text{ DNA homologous to λ DNA}}{\text{Fraction of λ DNA homologous to } E.\ coli \text{ DNA}}$$

$$\frac{3.1 \times 10^7}{X} = \frac{.002}{.33} \text{ and } X = 5.1 \times 10^9.$$

Cairns[10] finds a minimal molecular weight of 2.8×10^9 from results obtained by autoradiography of tritiated *E. coli* DNA.

In an analogous experiment in which the bacterial DNA immobilized in agar originated from *E. coli* K12 (λ) cells, which carry the λ prophage, a considerably larger fraction of the trapped DNA was found to be available for duplex formation with the λ DNA fragments. The results of this experiment are also shown in Figure 2. In this case 0.8 per cent of the embedded DNA is able to absorb the λ DNA.

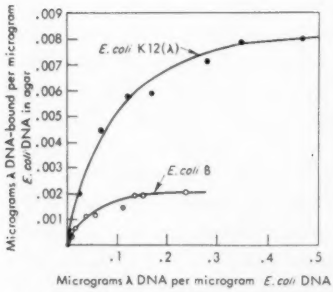

FIG. 2.—Reaction of λ DNA fragments with *E. coli* K 12 (λ) DNA (upper curve) and *E. coli* B DNA (lower curve). Increasing quantities of P^{32}-labeled λ DNA fragments were incubated overnight at 60°C with 0.1 gm agar containing either 17 μg *E. coli* K 12 (λ) DNA or 30 μg *E coli* B DNA.

Additional evidence indicating a difference in the binding of the λ DNA fragments to the DNA of the two strains of *E. coli* is shown in the following experiment. 0.01 μg of λ DNA was added to two DNA-agar preparations, one containing 150 μg *E. coli* B DNA, the other an equal quantity of *E. coli* K12 (λ) DNA. Both DNA-agar preparations were immobilized in 0.5 gm of agar. After a 10-hr incubation

period at 60°C, 70 per cent of the λ fragments were bound to the K12 (λ) DNA and only 28 per cent to that of the *E. coli* B DNA.

It is reasonable to assume that the 0.2 per cent of the *E. coli* B region is the same as a portion of an 0.8 per cent region in *E. coli* K12 (λ). The remainder (0.6 %) which can accept the labeled λ DNA fragments is presumably the λ DNA in the prophage state. This increment is precisely that expected if each *E. coli* nucleus had associated with it one complete molecule of λ DNA. Thus, the ratio of molecular weights of λ DNA to *E. coli* DNA is 6×10^{-3}.

Genetic relatedness of λ bacteriophage and other enterobacteria: It has been shown elsewhere that a quantitative measure of genetic relatedness among different enterobacteria can be made through duplex formation between two of the different DNAs or between the messenger RNA of one organism and the DNA of another.[2] Since a portion of the λ DNA molecule is related to a very specific segment of *E. coli* DNA, the opportunity exists for comparison of specific regions of two bacterial chromosomes. Thus, duplex formation between λ DNA and the DNA of bacterium other than *E. coli* demonstrates the presence of nucleotide sequences similar to 0.2 per cent of the *E. coli* genome.

Experiments were carried out in which labeled λ DNA fragments were incubated with DNA agar prepared from the DNA of several different bacteria. These DNA agar preparations were, in fact, aliquots of those used for reaction with *E. coli* DNA fragments and *E. coli* messenger RNA.[2] The fraction of λ DNA bound is recorded in Table 1 together with the amount of *E. coli* DNA fragments

TABLE 1

BINDING OF λ DNA FRAGMENTS TO VARIOUS DNA-AGAR PREPARATIONS

Organism	Source	Percentage of total λ DNA bound*	Relative percentage of *E. coli* DNA bound†
E. coli B	Laboratory strain	6.8	100
E. coli K 12 (λ)	"	9.5	101
E. coli K 12 (λ-sensitive)	"	5.8	tested
Salmonella typhimurium	ATCC 7823	8.2	Group A not 71
Shigella dysentariae/s	‡	13.5	71
Aerobacter aerogenes	ATCC 13048‡	11.1	48
Klebsiella pneumoniae	8821/6‡	0.7	25
Proteus vulgaris	ATCC 4669	0.2	14
Serratia marcescens	ATCC 4180	0.3	Group B 7
T4 bacteriophage	Laboratory strain	0.0	1

* 0.4 μgm fragmented, P^{32}-labeled, λ DNA was added to approximately 30 μg of the various bacterial DNAs trapped in agar.
† Data from McCarthy and Bolton (1963).[2]
‡ Cultures obtained through the generosity of Dr. M. Mandel, University of Massachusetts. Unless otherwise stated, all other bacterial cultures were obtained from American Type Culture Collection (ATCC).

bound relative to the homologous reaction. While the amounts of DNA in the agar vary somewhat so that the absolute percentage of λ DNA bound varies, all the values in excess of 1 per cent would represent homology. Those organisms most closely related to the *E. coli* show high reactions. On the other hand, the DNA of organisms such as *Klebsiella pneumoniae* and *Proteus vulgaris* showed no affinity for λ DNA despite sizeable cross reactions with *E. coli* DNA.

It is probable that the region of the λ DNA which is homologous to the *E. coli* DNA is also that segment which is complementary to the various DNAs tested. However, the results shown in Table 1 do not unequivocally allow this conclusion, since λ regions which showed no affinity might have been those which were capable

of duplex strand formation with the other DNAs. This possibility can be tested by first selecting only that λ DNA which is homologous to the *E. coli* DNA. Interaction of this selected material with the other DNAs would be a demonstration that it was only the "*E. coli* specific region" of the λ DNA which was involved. Such procedures would provide a possibility for the detailed comparison of nucleotide sequences among differing bacteriophage and bacterial chromosomes.

Discussion.—In the above paragraphs it has been shown that it is possible to calculate the molecular weight of the DNA of an organism by means of specific duplex formation with another DNA with which it has a partial homology. Since this calculation rests on the results of a number of different experiments, discussion of the data involved and the assumptions made in the calculation is appropriate at this point. Many of the principles involved are relevant not only in this context but also for other applications of the technique. The method rests wholly upon the high degree of specificity shown by reactions between DNAs of separate origin. That the reactions are specific is shown by the complete lack of reaction between *E. coli* DNA and DNA of mammalian origin, or between *E. coli* DNA and that of the virulent phage T2.[2]

Furthermore, the duplex formation is assumed to be quantitative as well as specific. This means that a given segment of DNA in the gel preparation will bind a molecule no larger than that segment. Clearly, if the DNA fragment bound were very much larger than the duplex region by which it paired to the immobilized DNA strand, the resulting free section could act as a receptor for more DNA fragments. Thus, the amount of free DNA bound would be greater than the amount of homologous DNA regions in the agar. This equivalence of bound DNA to the homologous region can only be approximately realized, since the lengths of the fragmented DNA molecules fall in a limited range and gaps will exist which are too small to be occupied by any of them. Moreover, a finite fraction of the trapped DNA molecules are presumably unavailable for base pairing by virtue of their involvement with the gel structure. Nevertheless, these effects cannot be large since the binding of *E. coli* DNA fragments to the homologous DNA-agar proceeds until an amount equivalent to some 80 per cent of the trapped DNA is fixed.[11] No further DNA is bound even when the added amount is increased several times. Thus, extension of the column by unpaired free regions is not observed.

Since essentially all of the trapped *E. coli* DNA is available to act as one partner of a duplex structure, the measurement of the maximum amount of λ DNA which can be bound must therefore be a reasonable estimate of the fraction of the total genetic sequences in *E. coli* DNA involved in this homology. Again, it is worth noting that the amount of λ DNA bound does not increase indefinitely, but shows real saturation characteristics. It should be made clear at this point that, although the duplex formation between λ DNA and *E. coli* DNA results from homology in base sequence, it is not possible to state how far these sequences are similar in detail or whether minor differences may exist.

About one third of the bacteriophage DNA molecule shows a relationship to a segment of the *E. coli* genome. This is also the fraction of the phage genome which is lost and replaced by a section of the bacterial genome in the transducing phage mutant λ dg.[4] This portion of the phage genome and the particular portion of the host chromosome may perhaps be described as allelic, although it is equally

certain that no regions allelic for the majority of the phage chromosome exist in the host. By the use of suitable mutants it should be possible to determine which of the phage genetic determinants are present in each of these two sections of the genome. The finding that the base composition of the λ DNA molecule is highly variable along its length might also be related to these two types of regions.[12]

In a more general sense, it would be of great interest to investigate other examples of virus-host genetic homology. Perhaps this type of relationship is common to all lysogenic bacteriophage or perhaps only to those which exhibit transduction. If it is a fairly general phenomenon, the use of a lysogenic bacteriophage DNA as a marker for a particular segment of the host molecule could contribute in molecular terms to the comparison of different bacterial species just as the transducing ability of these viruses has contributed to genetic mapping. Finally, the analogy which has often been made between lysogenic bacterial viruses and mammalian tumor viruses suggests extension of these investigations to these other possible examples of virus integration into the host chromosome.

Summary.—Measurement of the binding of bacteriophage λ DNA fragments to the DNA of *E. coli* demonstrated that a third of the phage genome was homologous to the bacterial chromosome. An amount equivalent to 0.2 per cent of the DNA of *E. coli* B was capable of duplex formation with λ DNA fragments. For *E. coli* K12 (λ) DNA, a value of 0.8 per cent was obtained. The difference between these values corresponds to the association of one complete molecule of DNA with the bacterial chromosome. λ DNA fragments also bind to DNAs of other bacteria related to *E. coli*.

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SEPARATION BY MONOCHROMATIC LIGHT OF PHOTOSYNTHETIC PHOSPHORYLATION FROM OXYGEN EVOLUTION

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Although photoproduction of oxygen is an essential part of the over-all photosynthetic process in plants, it does not appear to be essential for the formation of ATP,[1] a key step in the conversion of light energy into chemical energy.[2–4] Upon illumination, isolated chloroplasts form ATP by two photochemical reactions, cyclic and noncyclic photophosphorylation.[4, 5] In neither of these is photoproduction of oxygen essential: it is either absent, as in the case of cyclic photophosphorylation,[4, 6] or, when present, as in the case of noncyclic photophosphorylation,[7, 8] it can be experimentally separated[9] without impairing ATP formation (and TPN reduction). From this, it was suggested[10, 11] that the photosynthetic energy conversion process consisting of cyclic and noncyclic photophosphorylation is basically the same in oxygen-evolving green plants as in the strictly anaerobic photosynthetic bacteria, the photosynthesis of which is never accompanied by oxygen evolution. Oxygen evolution would, in this view, be an accessory photochemical reaction[10] dependent mainly on an accessory pigment system, which like chlorophyll *b* or the phycobilins, is found only in oxygen-evolving photosynthetic cells.

Other investigators have suggested, however, that ATP formation by chloroplasts *in vivo* is always accompanied by oxygen evolution. In this view, the experimentally demonstrable cyclic photophosphorylation without oxygen evolution would be an artifact resulting from the use of such added cofactors as methyl phenazonium methosulfate (phenazine methosulfate); in endogenous cyclic photophosphorylation, it was held,[12] oxygen evolution would always occur but would be masked by a simultaneous and equal oxygen consumption, the over-all result being an oxygen exchange, i.e., a pseudocyclic[6] type of photophosphorylation.

It seemed desirable, therefore, to investigate the relation of ATP formation to oxygen evolution in the recently discovered endogenous type of cyclic photophosphorylation by chloroplasts,[13] the type dependent on the native chloroplast protein, ferredoxin. This cyclic photophosphorylation is free from the objection that it is catalyzed by exogenous catalysts, some of which, like phenazine methosulfate, are clearly nonphysiological.

The findings reported here indicate that the ferredoxin-dependent cyclic photophosphorylation by isolated chloroplasts is independent of photoproduction of oxygen; it occurs in far-red monochromatic light (708 mμ) at which only little oxygen evolution by isolated chloroplasts has been observed. The experimental unmasking of ferredoxin-dependent cyclic photophosphorylation in chloroplasts was greatly facilitated by the prevention of photochemical oxygen evolution; nevertheless, this type of photophosphorylation is facilitated, not impeded, by the presence of oxygen gas, which always surrounds functioning chloroplasts *in vivo*. These findings are consistent with the central role which is envisaged[13] for ferredoxin in photosynthetic phosphorylation (cyclic and noncyclic) by chloroplasts in intact cells.

Methods.—The source of light was an incandescent 1200-watt General Electric projection lamp, cooled by a combination of rapid forced air circulation, circulation of cold water through copper coils wound around the metal lamp housing, and by passing the light beam through a glass-enclosed circulating cold water filter, 5 cm thick. The monochromatic spectral bands were isolated with interference filters (made by Balzers Aktiengesellschaft, Balzers, Liechtenstein) and focused on the bottom of the reaction vessel by a combination of lenses and a 45° mirror, an arrangement similar to that described by Warburg and Krippahl.[14] The spectral characteristics of interference filters were checked with a Cary Model 14 recording spectrophotometer. The purity of the isolated monochromatic band was spectroscopically monitored. In using monochromatic light at 708 mμ (or longer) it was important to select filters with sharp cutoff characteristics toward radiation on the shorter wavelength side.[15] Without these precautions even a relatively small admixture of shorter wavelength radiation may nullify the effects of far-red monochromatic light.

The intensity of the incident monochromatic light was measured by Warburg's bolometric method,[16] with the use of a Lumner-Kurlbaum bolometer. The bolometer was calibrated against a standard lamp from the U.S. Bureau of Standards. The incident light intensity in all experiments was greater than 3 μeinsteins ("μmoles" of quanta) per min. At lower light intensities little photophosphorylation was observed. The light intensity used was adequate to give significant but not high photophosphorylation rates.

The photophosphorylation reaction was carried out at 15°C in rectangular Warburg vessels, which were continuously shaken in a rectangular constant temperature waterbath with a glass side wall through which the light beam entered to fall on the 45° mirror that was mounted in the bath below the reaction vessels. Broken chloroplasts[17] (P_{1s} or C_{1s}) were used in all experiments. The ATP formed was estimated by the method of Hagihara and Lardy,[18] including the step with the addition of K_2HPO_4 as a carrier. Oxygen evolution was measured polarographically with a platinum electrode modeled after that of Hagihara.[19]

Results and Discussion.—In previous experiments[13] with white light under anaerobic conditions, the ferredoxin-dependent cyclic photophosphorylation was experimentally demonstrated by omitting TPN and by using the inhibitor CMU,[1] noted for its effectiveness in inhibiting oxygen evolution by chloroplasts. In other experiments, not reported here, the omission of chloride gave results similar to those obtained by the addition of CMU. (Without chloride, chloroplasts cannot evolve oxygen.[20, 21]) These findings indicated that other suitable experimental methods for suppressing photoproduction of oxygen by chloroplasts should also be effective in unmasking the ferredoxin-dependent cyclic photophosphorylation.

In photosynthesis by intact cells, far-red monochromatic light does not support oxygen evolution[22] and, at wavelengths longer than 680 mμ, is associated with a decline in quantum efficiency.[23] In our earlier experiments with isolated chloroplasts, in which the oxygen evolution reaction was chemically separated from ATP formation and TPN reduction, there were indications that these partial reactions may lend themselves to separation by monochromatic light.[9] It seemed possible therefore that far-red light of a wavelength that would not support oxygen evolution would still support the ferredoxin-dependent cyclic photophosphorylation and do

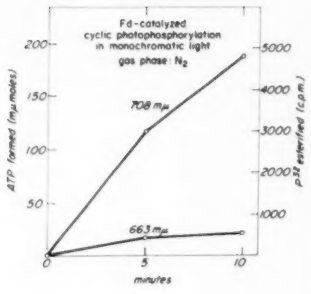

FIG. 1.—Ferredoxin-catalyzed cyclic photophosphorylation in monochromatic light. The reaction mixture (final volume of 3.0 ml) included broken chloroplasts containing 400 μg of chlorophyll, 2 mg of spinach ferredoxin, and the following in μmoles: tris buffer, pH 8.3, 100; $MgCl_2$, 5; ADP, 2; $K_2HP^{32}O_4$, 2. Prior to adding $K_2HP^{32}O_4$, each reaction mixture was preilluminated for 5 min by its respective monochromatic light. Light intensity was 5.8 and 4.1 μeinsteins per min at 708 mμ and 663 mμ, respectively. The reaction vessels were capped with rubber serum bottle stoppers through which, at the indicated times, 0.5 ml aliquots of the reaction mixture were withdrawn with a syringe, for ATP determination. The ATP formed is plotted as μmoles ATP in 1.5 ml of reaction mixture. The reaction vessels were continuously flushed with N_2, first for 5 min in the dark, and then during the entire period of exposure to light.

so without the addition of a chemical inhibitor of oxygen evolution.

These considerations were sustained by experiments represented by Figure 1 and Table 1. Table 1 shows that red monochromatic light effectively supported oxygen evolution by isolated chloroplasts at wavelengths below but not above 700 mμ. Conversely, as shown in Figure 1, ferredoxin-dependent cyclic photophosphorylation, under anaerobic conditions and without added CMU, occurred at 708 mμ but was greatly reduced at 663 mμ. In other experiments, similar to those with white light,[13] ferredoxin-dependent anaerobic cyclic photophosphorylation was strikingly increased at 663 mμ by the addition of CMU which abolished photoproduction of oxygen at this wavelength (Table 1).

TABLE 1

EFFECT OF RED MONOCHROMATIC LIGHT ON PHOTOPRODUCTION OF OXYGEN BY ISOLATED CHLOROPLASTS

Wavelength (mμ)	O_2 evolved (μmoles/min)
663	0.14
672	0.12
696	0.11
708	0.03
718	0.01
663, CMU	0.00

The reaction mixture (final volume 2.7 ml) included chloroplasts (containing 180 μg of chlorophyll), 150 μg of spinach ferredoxin, and the following in μmoles: tris buffer, pH 8.3, 125; $MgCl_2$, 5; TPN, 1. 0.5 μg of CMU was added where indicated. The same reaction mixture was successively illuminated by each respective beam of monochromatic light for about 30 sec. The optical system was adjusted to give the same intensity of monochromatic light (5.2 μeinsteins/min) at each wavelength of light. Temperature was 17°C. The initial concentration of O_2, dissolved in the reaction mixture, was 6.1×10^{-5} M.

The inability of monochromatic light at 663 mμ to support *anaerobic* cyclic ATP formation (Fig. 1) was observed after preilluminating the chloroplast system at this wavelength for 5 min under nitrogen and in the absence of CMU. The significance of these observations, which led to another series of experiments, will be discussed elsewhere.

Since reduced ferredoxin is nonenzymatically oxidized by molecular oxygen,[24] it might be argued that photochemically reduced ferredoxin would constitute an electron leak to oxygen and thus be ineffective in catalyzing cyclic photophosphorylation under aerobic conditions, that is, under conditions which normally exist around chloroplasts *in vivo*. This possibility was tested by investigating cyclic photophosphorylation at 708 mμ, under aerobic conditions. Figure 2 shows that at 708 mμ ferredoxin catalyzed ATP formation in an atmosphere of air. In fact, under aero-

bic conditions, low concentrations of ferredoxin were more effective than under anaerobic conditions in catalyzing ATP formation (compare Fig. 2 here with Table 1 in ref. 13, which gives the ATP formed in 15 min of illumination).

Evidence that the photophosphorylation at 708 mμ shown in Figure 2 was indeed of the ferredoxin-dependent cyclic type was provided by experiments with antimycin A. Sensitivity to low concentrations of antimycin A was found to distinguish the ferredoxin-dependent cyclic photophosphorylation from other types of photophosphorylation by chloroplasts: cyclic photophosphorylation catalyzed by phenazine methosulfate or menadione, and the ferredoxin-dependent noncyclic photophosphorylation coupled with TPN reduction. Figure 3 shows that aerobically at 708 mμ, as under N_2 in white light,[13] antimycin A inhibited ATP formation.

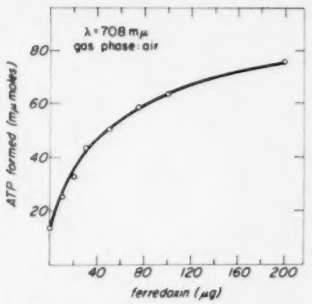

FIG. 2.—Effect of ferredoxin on cyclic photophosphorylation in air at 708 mμ. The reaction mixture contained (in a final volume of 1.5 ml) spinach ferredoxin as indicated, broken chloroplasts containing 200 μg of chlorophyll, and the following in μmoles: tris buffer, pH 8.3, 50; $MgCl_2$, 2.5; ADP, 1; $K_2HP^{32}O_4$, 1. The reaction was carried out for 4 min under air. The light intensity was 4.9 μeinsteins per min.

Heretofore, a notable distinction between oxidative phosphorylation by mitochondria and photosynthetic phosphorylation by chloroplasts was the insensitivity of the latter to low concentrations (5×10^{-5} *M*) of 2,4-dinitrophenol (cf. review[25]). However, as shown in Figure 4, ferredoxin-dependent cyclic photophosphorylation was strongly inhibited by low concentrations of dinitrophenol. Fifty per cent inhibition was given by about 3×10^{-5} *M* dinitrophenol.

Apart from sensitivity to antimycin A and dinitrophenol, ferredoxin-dependent cyclic photophosphorylation differed from other photophosphorylations by our chloroplast preparations in that it occurred at 708 mμ. The ferredoxin-dependent noncyclic photophosphorylation (with water as the electron donor) failed to take place at this wavelength. Under our experimental conditions, we also found no cyclic photophosphorylation at 708 mμ in the presence of phenazine methosulfate with the same monochromatic beam which was effective for the ferredoxin-dependent cyclic photophosphorylation. However, a cyclic photophosphorylation at 710 mμ, catalyzed by phenazine methosulfate, has been reported by Kok and Hoch.[26]

Summary.—To unmask the ferredoxin-dependent cyclic photophosphorylation and to demonstrate its independence from photoproduction of oxygen it was necessary to use special experimental devices such as far-red monochromatic light, anaerobic conditions, and inhibitors of oxygen evolution. However, if ferredoxin-dependent cyclic photophosphorylation is to be considered the cyclic photophosphorylation *in vivo*, then it must be shown that this type of photophosphorylation will also occur under the natural conditions of photosynthesis in leaves, i.e., in white light and in the presence of oxygen. The present findings and those reported earlier[13] provide evidence that ferredoxin-dependent cyclic photophosphorylation by isolated chloroplasts is indeed compatible with these natural conditions of photosynthesis in leaves. Evidence for cyclic photophosphorylation in intact leaves, as distinguished from isolated chloroplasts, has recently been reported by Forti and Parisi.[27]

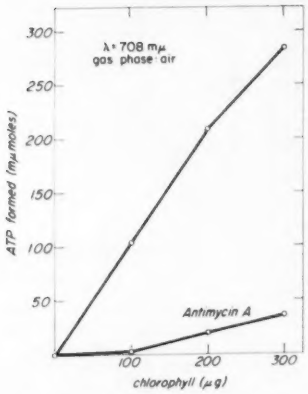

FIG. 3.—Effect of antimycin A on ferredoxin-catalyzed cyclic photophosphorylation at 708 mμ under air. The reaction mixture was as given for Fig. 2, except that 300 μg of spinach ferredoxin was used throughout and the amount of chlorophyll varied as indicated. 10 μg of antimycin A were added where indicated. The reaction was carried out for 10 min. Light intensity was 3.8 μeinsteins per min.

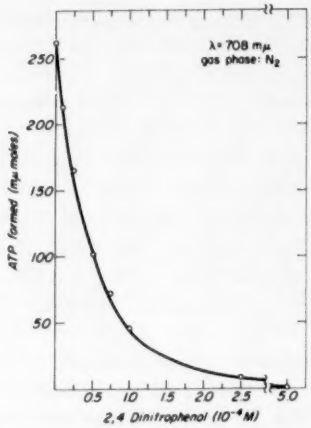

FIG. 4.—2,4-Dinitrophenol inhibition of ferredoxin-catalyzed cyclic photophosphorylation. The reaction mixture was as given for Fig. 2, except that 1.5 mg of spinach ferredoxin was used throughout, and a variable amount of 2,4-dinitrophenol as indicated. The reaction was carried out for 5 min under nitrogen. Light intensity was 4.1 μeinsteins per minute at 708 mμ.

Work from several laboratories (most recently reviewed by Smith and French[28]) has led to a now widely held view that photosynthesis in green plants involves the cooperation of at least two pigment systems, each of which carries on separate partial reactions essential to the over-all process. There is little biochemical evidence, however, as to which partial photochemical reaction is associated with which specific pigment system. The occurrence of ferredoxin-dependent cyclic photophosphorylation at 708 mμ and its independence from oxygen evolution is consistent with a hypothesis that, in chloroplasts, photophosphorylation (and TPN reduction[9, 10]) is dependent mainly on the chlorophyll *a* pigment system and hence can be experimentally separated from the photochemical activity of chlorophyll *b*, which is associated with photoproduction of oxygen.

One of us (Arnon) wishes to thank Professor Otto Warburg for the hospitality of his Institute where as a guest investigator in 1955–1956 he learned the optical and photometric techniques used in this investigation. We thank Professor J. E. Gullberg for advice, and Lars O. Björn for assistance in the initial assembly and testing of the optical apparatus. Richard K. Chain, Berah D. McSwain, and William Ufert have given excellent technical assistance with the current experiments.

* Aided by grants from the U.S. Public Health Service and the Office of Naval Research.

[1] Abbreviations: ATP, adenosine triphosphate; ADP, adenosine diphosphate; P_i, orthophosphate; TPN, $TPNH_2$, oxidized and reduced forms of triphosphopyridine nucleotide; CMU, *p*-chlorophenyldimethyl urea.

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*CONFORMATIONAL CHANGES IN MYOSIN B AND ACTOMYOSIN INDUCED BY ATP**

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Communicated by Julius H. Comroe, Jr., July 12, 1963

The ATP superprecipitation of "myosin B" suspended in neutral 0.1 *M* KCl depends on many conditions in just the same way that muscle contraction depends on the same conditions; the phenomenon is therefore considered to be the simplest model of contraction. Our investigations seek to ascertain the molecular nature of this primitive "contraction," as well as of other ATP-myosin B reactions. The materials we have used—myosin, actin, myosin B—were extracted from rabbit muscle and purified (more stringently than usual) by standard procedures.[1] Also the methods employed—light scattering, viscosity, electron microscopy (chiefly by the mica replica technique[2, 3])—were employed in conventional fashion. It should be noted, however, that while most physical studies on muscle proteins employ KCl solutions, it was necessary for electron microscopy to use NH_4HCO_3 solutions;

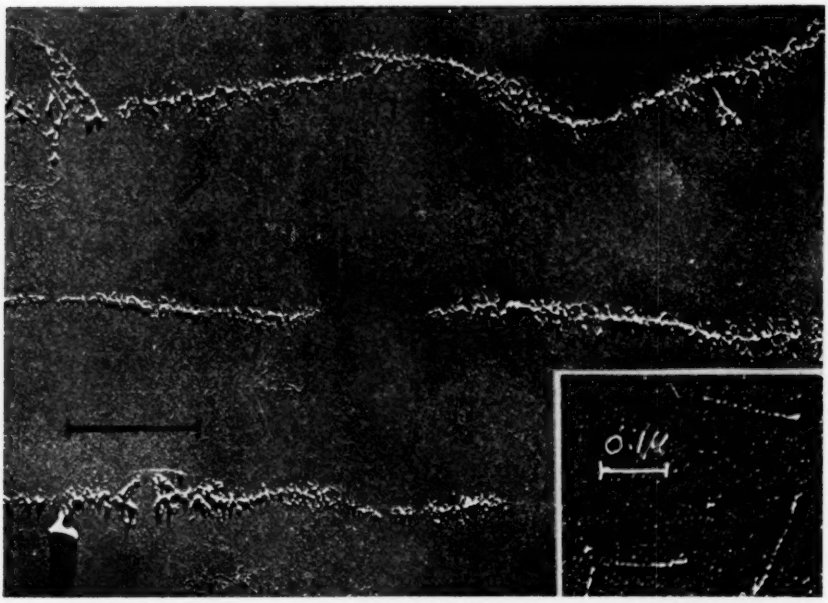

FIG. 1.—Main area: Myosin aggregates oriented by streaking. × 37,600. Insert: Individual myosin molecules. × 100,000. (Note: Scale line is 0.5 μ except where noted. All electron micrographs shown are of specimens prepared by the C-Pt mica replica technique.)

FIG. 2.—F-actin. × 121,000. Filament width, about 80 A. Arrow indicates strand separation.

physical studies in both media indicated that the reactions to be reported were not significantly affected by this substitution.

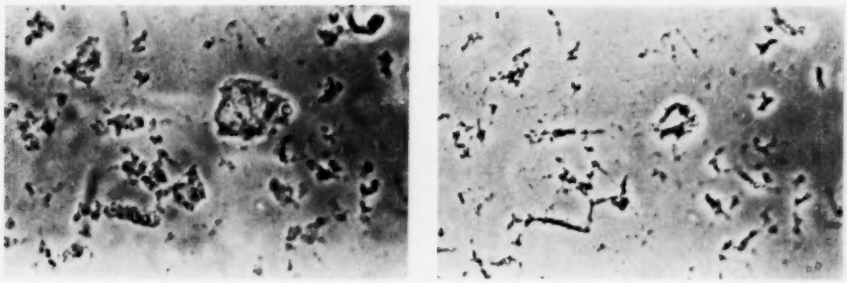

FIG. 3.—Somewhat flattened particles of myosin B suspended in 0.09 *M* KCl, *ca.* × 900. *Left:* control; *right:* with 10^{-3} *M* ATP.

The myosin molecule is a thin rodlet, a little over 0.1 μ long (Fig. 1, insert), with one bulbous end (on fragmentation this end constitutes the H-meromyosin and contains the ATPase site.)[4,5] Myosin sometimes aggregates into long, smoothly contoured structures showing no nodulation.[5] In the present work it has been found that myosin suspensions oriented by streaking on freshly cleaved mica surfaces also form irregularly contoured aggregates (Fig. 1).

F-actin is now known (Ref. 6 and Fig. 2) to be a "rope" of more than one strand of spheroidal actin monomers wound together helically; Figure 2 shows that the number of strands may exceed 2, and that the ropes or filaments are potentially endless; in some areas (Fig. 2) individual monomers are discernible (50 A nodulation).

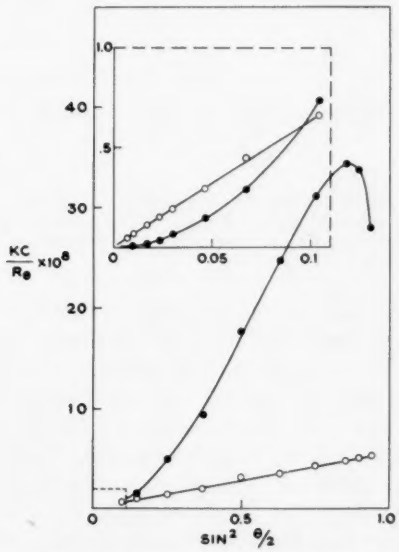

FIG. 4.—Zimm plot for a myosin B suspension without ATP (open circles) and with m*M* ATP (filled circles). Protein 0.8×10^{-3}%, in 0.06 *M* KCl and 0.02 *M* Tris maleate, pH 7.0. Top graph is enlargement of dotted-in area in bottom graph.

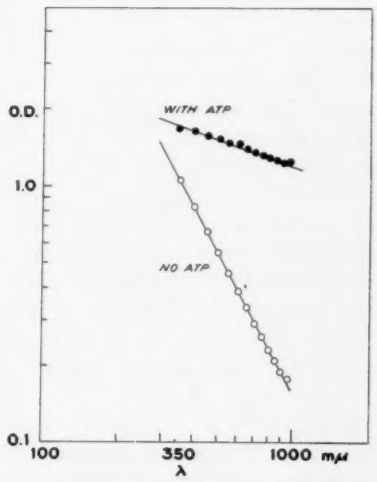

FIG. 5.—Logarithmic plot of O.D. versus wavelength for myosin B without ATP (open circles) and with mM ATP (filled circles). Protein, 0.01% in 0.06 *M* KCl and 0.02 *M* Tris maleate, pH 7.0.

F-actin is known to complex with myosin, forming "actomyosin." It is generally thought that, in its reactions, actomyosin is qualitatively identical to "myosin B" extracted from muscle as such. At neutral pH the solubility of either myosin B or actomyosin as a function of electrolyte concentration closely resembles the solubility of myosin.[7] If the electrolyte is KCl (the behavior in NH_4HCO_3 is similar), there is, at *ca.* 4°C, a solubility minimum near 0.08 *M;* at 0.60 *M* both systems are entirely dissolved. With rather misleading emphasis, these conditions are commonly called "low ionic strength" and "high ionic strength," respectively. At low ionic strength the systems are flocculent suspensions (by appropriate maneuvers macro-threads can be readily prepared). At a sufficiently high protein concentration, addition of mM ATP causes the suspension to synaerese into a clot (or the macro-thread to contract); in more dilute suspensions phase microscopy shows that irregular swelled particles contract (Fig. 3). To study the behavior of the "average" particle, however, light scattering methods are superior. Figure 4 shows a "Zimm plot" of scattered light intensities as a function of scattering angle for a very dilute (and thus more stable) suspension of myosin B, without and with ATP. At small angles it is clear that ATP brings about an increase in scattered light. While there is no satisfactory theory for the scattering from such large, irregular particles, the foregoing response is qualitatively expected if ATP causes the particles to become more dense. Figure 5 shows the transmission optical density (OD) as a function of wavelength (for nonabsorbed wavelengths)[8] for a similar suspension without and with ATP. Clearly, ATP addition causes an increase in total light scattered as well as a more marked deviation from the inverse fourth-power wave-

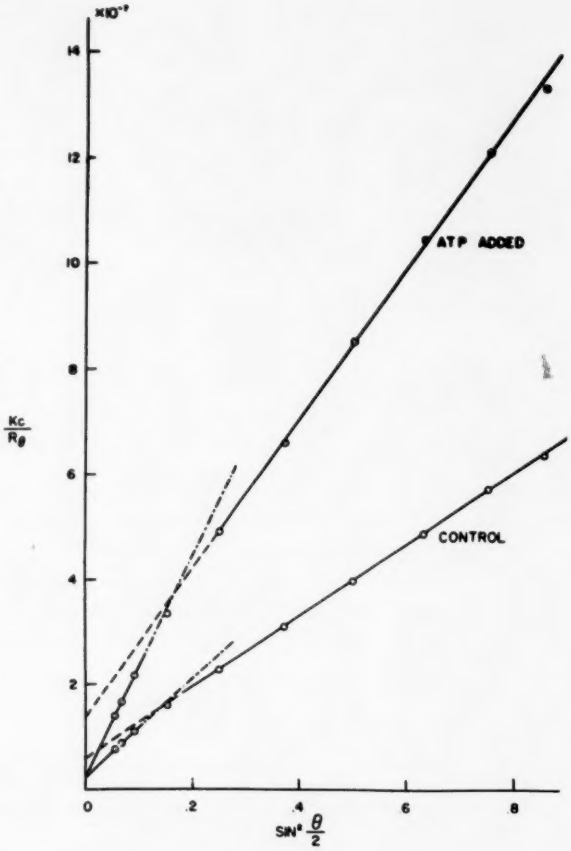

FIG. 6.—Zimm plot for a myosin B solution without ATP (lower curve) and with mM ATP. Protein, 0.01%, in 0.60 *M* KCl, and 0.10 *M* histidine, pH 7.0. Reproduced by permission of the *J. Am. Chem. Soc.*

length dependence. The result of ATP addition at "high ionic strength" has been widely thought to be dissociation of the actomyosin complex into F-actin and myosin. Our studies of the angular dependence of light scattered from myosin B solutions, however, first suggested[9] that ATP causes the particles to *expand*, at constant weight-average molecular weight. This conclusion was assured by our re-examination of the problem[1] (as well as by the studies of another laboratory[10]) which showed (Fig. 6 reproduced from ref. 1) that ATP certainly expands the larger particles (molecular weights, *ca.* 10^7 gm; radii of gyration of several tenths of a micron), and that such dissociation as occurs among smaller particles is largely into myosin. At high ionic strength the transmission optical density decreases *uniformly* at all wavelengths when ATP is added. The OD-λ relationship can be studied over the whole range of ionic strengths (Fig. 7). In summary of the ATP-myosin B reactions, we may say: at neutral pH and 0.08 *M* KCl, visual observation as well as the angular dependence of scattered light intensity shows that ATP addition causes particles of myosin B to *contract*, and the logarithmic slope of the OD-λ relationship to decrease. At 0.60 *M* KCl, the angular dependence of scattered light intensity shows that ATP addition causes particles of myosin B to *expand* (and some particles to dissociate off myosin), but does not change the logarithmic slope of the OD-λ relationship. If decrease of the logarithmic slope indicates contraction, then it is suggestive that the magnitude of this decrease as a function of ionic strength (Fig. 7) follows closely the corresponding solubility curve of myosin.[7]

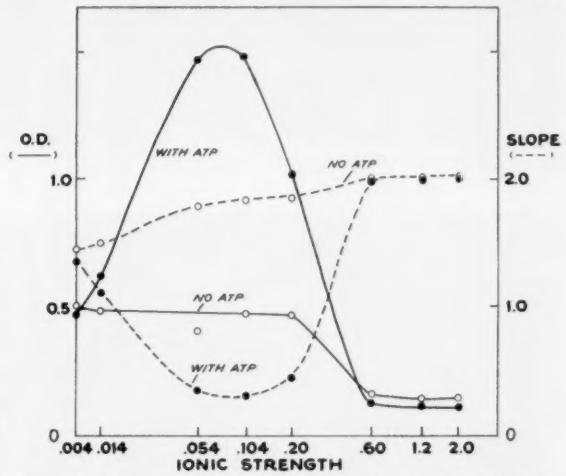

FIG. 7.—Left ordinate: $OD_{550m\mu}$. Right ordinate: Logarithmic slope of OD versus wavelength relation. These ordinates are plotted against [KCl]. Myosin B, 0.01% in 0.02 *M* Tris maleate. Open circles, no ATP; closed circles, mM ATP.

The results to this point show that in a myosin B system there exist some aggregates—possibly coaggregates of myosin and actin—whose structure is altered by ATP, and the results map the dependence of this alteration on relevant parameters. But the resolution provided by the light-scattering methods is insufficient to tell how myosin and actin participate in these alterations. In particular, these results shed no light on the current question of whether the dimensional changes of the particles are produced by the relative translation of two rigid moieties ("sliding" motion), or by changes in configuration of one moiety ("curling" motion). To explore these issues further we have used electron microscopy.

When myosin B or actomyosin solutions have been streaked on freshly cleaved mica surfaces, we have found just one coaggregate of actin and myosin. This structure resembles a ladder in that a dense myosin aggregate ("rungs") lies between two

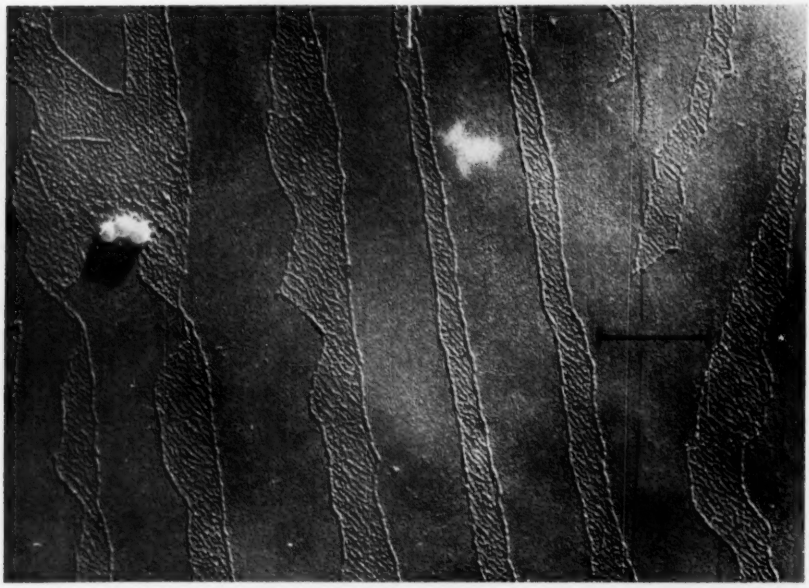

FIG. 8.—Ladder-like structures prepared from myosin B in 0.70 *M* NH_4HCO_3 suspension. ×31,900.

FIG. 9.—Expanded ladder prepared from myosin B in 0.70 *M* NH_4HCO_3 and 10^{-5} M ATP solution. ×5,300. Arrow marks abrupt change in diameter of F-actin rail.

roughly parallel F-actin filaments ("rails") (Fig. 8); streaked out, the ladder is a few tenths of a micron wide and may be many micra long. This identification of components is morphological, i.e., the rails are long, smoothly contoured filaments showing some 50 A nodulation, while the rungs are a very irregularly contoured aggregate, closely resembling that shown in Figure 1. On the occasions in which they have been used, other techniques (viz., sprayed droplets of macro-drops rather than streaking; negative staining[11] rather than mica replica; fixation by freezing) have also shown ladders. Streaking, however, seems to be important in revealing ladders, suggesting that in aqueous suspension the ladders may be folded or stacked in flat structures sometimes seen by phase microscopy of suspensions.

The preparations used for EM studies of reactions have always been made at such times after ATP addition, and under such ionic conditions that separate light scattering observations showed the ATP response to be maximal. When ATP is

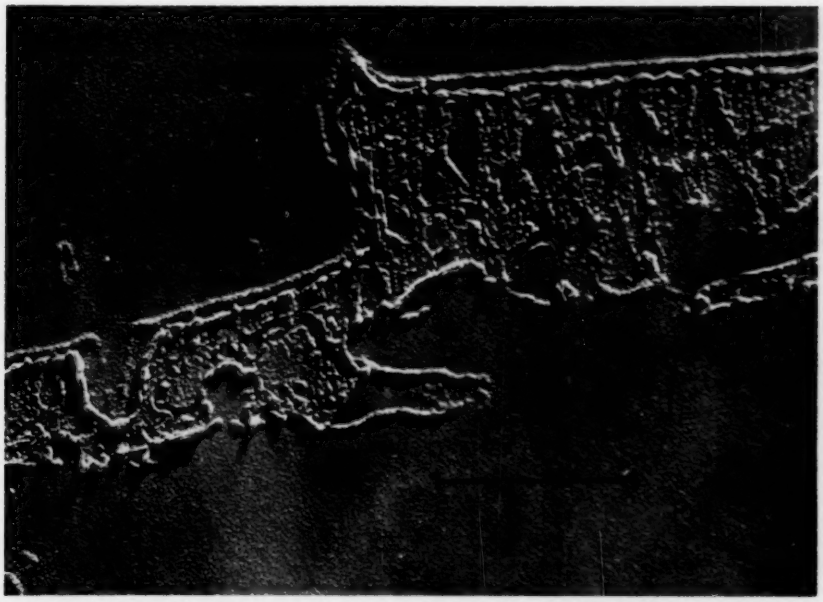

FIG. 10.—Contracted ladder prepared from myosin B in 0.09 *M* NH_4HCO_3 and 10^{-5} *M* ATP suspension. ×59,400. Arrow marks typical invagination.

FIG. 11.—Contracted ladder prepared by freezing-drying from myosin B in 0.09 *M* NH_4HCO_3 and 10^{-5} *M* ATP suspension. ×20,600.

added at high ionic strength, the ladders extend by a thinning out of the myosin rungs with consequent separation of the rails (Fig. 9); under these circumstances some "free" actin and myosin are occasionally seen. Observation after ATP has been added at low ionic strength has proved more difficult, but whenever a ladder has been recognized, it has invariably been curled up, i.e., with thickened contorted rails and with a reduced width due to a condensation of the myosin (Fig. 10). Although fraught with some dangers (the freeze-drying operation itself, without ATP, induces some changes similar to those caused by ATP, presumably because dehydration is involved in both processes), the freeze-drying technique has

FIG. 12.—Expanded ladder prepared from myosin B in 0.70 *M* NH_4HCO_3 and 10^{-4} *M* ATP solution. ×31,300. Arrows mark stubs still attached to rails at oblique angles.

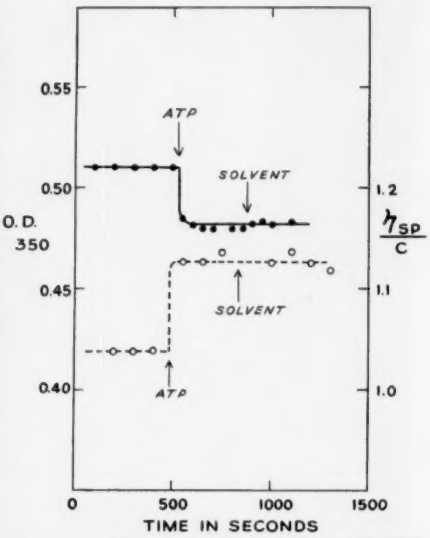

FIG. 13.—Changes induced by *ca.* 2 mM ATP in a 0.21% suspension of myosin in 0.05 *M* KCl and 0.05 Tris maleate, pH 7.0, 25°C. Left-hand scale and full line: $OD_{350m\mu}$. Right-hand scale and dotted line: η_{sp}/c.

provided the best pictures of contracting ladders (Fig. 11).

Taking light scattering and EM observations together, we conclude that at both high and low ionic strengths there exist ladder-like structures of F-actin and myosin. ATP at high ionic strength extends them (and somewhat dissociates them, but chiefly into myosin); at low ionic strength it contracts them by a change in configuration of the actin-myosin structure. No EM picture, by any technique, has ever suggested contraction by relative translation: the configurational change has always been obvious.

In some instances in which ladders have been extended by ATP, the junctures of the myosin with the F-actin rails are clearer, and it is seen (Fig. 12) that the myosin rodlets are not perpendicular to the F-actin axis. Under these conditions the rails are *ca.* 80 A wide. Sometimes rails have myosin stubs attached every 130 A along the F-actin filament, recalling the cross bridges found in thin sections of muscle.[12]

The question of whether on ATP addition the force is generated in the myosin rungs and is transmitted to the actin or vice versa is moot. Noting that the rungs are actually a rather concentrated *myosin* system, we have examined the first alternative by asking whether, under the ionic conditions of superprecipitation, ATP or pyrophosphate (PP) induce any structural changes in concen-

trated myosin rigorously free of actomyosin response. The polyphosphates do induce such changes. Figure 13 illustrates the changes with ATP; the changes induced by PP are very similar. It may be, however, that the myosin molecules directly joined to the rails play a special role, since on ATP addition the rails are observed to thicken.

The present work does not preclude that these same actin and myosin components, undergoing these same force interactions, but in a different geometrical arrangement, could bring about the relative translation featured in a popular theory of muscle contraction.[12] However, it is clear that the behavior of this simple model suggests that contraction is achieved by configurational change, not sliding.

The authors are deeply indebted to many colleagues from the EM laboratory of the Mellon Institute and from the Cardiovascular Research Institute, but special thanks are due to Professor Shizuo Watanabe for continuous advice, and to Dr. Carolyn Piel for generously providing EM facilities in the University of California.

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§ Career investigator, American Heart Association.

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ON THE ABSOLUTE STABILITY OF SAMPLED-DATA CONTROL SYSTEMS*

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Communicated by S. Lefschetz, July 31, 1963

1. Let $\varphi = \varphi(\sigma)$ be a continuous real scalar function of the real variable σ with continuous and bounded derivative and such that

$$\varphi(0) = 0 \quad 0 < \sigma\varphi(\sigma) \leqq \sigma^2 k, \; k < +\infty. \tag{1}$$

Consider the sampled-data control system Ω which consists of a linear and time invariant part and the nonlinearity $\varphi = \varphi(\sigma)$. Such a system will be represented by the difference equation

$$\begin{aligned} x_{h+1} &= Ax_h - a\varphi(\sigma_h) \\ \sigma_h &= 2b'x_h, \; h = 0,1, \ldots \end{aligned} \tag{2}$$

where A is a real $n \times n$ matrix, and a and b are real vectors.

We shall assume that the matrix A is stable, that is, that eigenvalues γ_i satisfy

$$|\gamma_i| < 1, \qquad i = 1,2, \ldots, n, \tag{3}$$

that the linear part of Ω is completely controllable[1] (the vectors $a, Aa, \ldots, A^{n-1}a$ are linearly independent) and completely observable[1] (the vectors $b, A'b, \ldots, (A')^{n-1}b$ are linearly independent), and also that the system (2) is asymptotically stable for all linear $\varphi(\sigma_h) = \sigma_h m$ in the class (1). Thus, the system (2) has only one equilibrium point: $x_h = x_{h+1} = 0$.

2. One wants to derive a sufficient condition for the existence of a Liapunov function of the type

$$v = x'Hx + \beta\int_0^\sigma \varphi(s)ds \quad H > 0 \tag{4}$$

which proves global asymptotical stability for the system (2) with respect to all the nonlinearities (1) (absolute stability). Thus, we look for a solution of the Lur'e problem[2-5] for (2).

The v-difference of (4) along the solutions of (1) is:

$$\Delta v = x_h'[A'HA - H]x_h - 2\varphi(\sigma_h)x_h'A'Ha + \varphi^2 a'Ha + \beta\int_{\sigma_h}^{\sigma_{h+1}} \varphi(s)ds. \tag{5}$$

By the mean value theorem, since $\varphi(\sigma)$ is continuous with continuous and bounded derivative, we have:[6]

$$\beta\int_{\sigma_h}^{\sigma_{h+1}} \varphi(s)ds \leqq \beta\varphi(\sigma_h)[\sigma_{h+1} - \sigma_h] + \frac{\mu}{2}\beta \operatorname{sign}\beta[\sigma_{h+1} - \sigma_h]^2 \tag{6}$$

where

$$\mu > \left.\frac{d\varphi}{d\sigma}\right|_{max} > 0. \tag{7}$$

By adding to the scalar function (5) the identity

$$-\alpha[\varphi(\sigma_h)\sigma_h - 2\varphi(\sigma_h)x_h'b] = 0, \tag{8}$$

and taking into account the inequality (6), one obtains:

$$\Delta v \leqq x_h'[A'HA - H + rr']x_h - 2\varphi x_h'[A'Ha - \alpha b - \beta d] - \varphi^2[\alpha \frac{1}{k} + 2\beta\xi - a'Ha] - \alpha\varphi[\sigma_h - \frac{1}{k}\varphi] \quad (9)$$

where r, d, and ξ are defined, respectively, by:

$$r = \sqrt{2\mu|\beta|}\,(A - I)'b \quad (10)$$

$$d = -(2\mu\, b'a \operatorname{sign}\beta - 1)\,(A - I)'b \quad (11)$$

$$\xi = a'b - \mu(a'b)^2 \operatorname{sign}\beta. \quad (12)$$

The expression Δv will be negative definite along the solutions of (2) if there exists a real scalar γ, a real vector q, and a real matrix $H = H'$ such that

$$A'HA - H + rr' + qq' = 0,$$

$$A'Ha - \alpha b - \beta d = \gamma q,$$

$$2\beta\xi + \alpha\frac{1}{k} - a'Ha = \gamma^2 > 0. \quad (13)$$

Then the expression (9) becomes:

$$\Delta v \leqq -[q'x + \gamma\varphi]^2 - \alpha\varphi[\sigma_h - \frac{1}{k}\varphi]. \quad (14)$$

By using the same procedure as in reference 5, one can show that:

LEMMA. *Necessary and sufficient conditions for the existence of γ, q, H, as above are*

$$\alpha\frac{1}{k} + 2\beta\xi + 2\,Re\{(\alpha b + \beta d)'(Iz - A)^{-1}a\} - |r'(Iz - A)^{-1}a|^2 \geqq 0 \quad (15)$$

$$\alpha\frac{1}{k} + 2\beta\xi > 0 \quad (16)$$

for all z ($z = e^{i\omega}$) and some real numbers α and β.

By some algebraic manipulations one may show that (15) and (16) can be written in the following simpler form:

$$(\gamma + \beta)\frac{1}{k} + Re(\gamma + \beta z)W(z) - \frac{\mu|\beta|}{2}|(z - 1)W(z)|^2 \geqq 0, \quad (17)$$

$$\gamma = \alpha - \beta \quad (18)$$

where

$$W(z) = 2b'(Iz - A)^{-1}a \quad (19)$$

is the open-loop transfer function of the linear part of (2), and

$$(\gamma + \beta)\frac{1}{k} + 2\beta\xi > 0. \quad (20)$$

Thus,

THEOREM. *If the conditions* (17) *and* (20) *are satisfied for all* z *such that* $|z| = 1$ *and for real numbers* γ, β *such that*

$$\gamma + \beta \geqq 0 \tag{21}$$

then the system (2) *is absolutely stable.*

Proof: Let us show that Δv is negative definite along the solutions of (2). When $\gamma + \beta = 0$ or $\varphi(\sigma) = k\sigma$, the inequality (14) becomes

$$\Delta v \leqq - [q'x - \gamma\varphi]^2. \tag{22}$$

From (6) one can see that in (22) the equality sign may occur only if $\sigma_{h+1} = \sigma_h$, that is, $2b'(x_{h+1} - x_h) = 0$, that is, (because of c.o. of the system (2)) if $x_{h+1} = x_h$, that is, on equilibrium points. But the only equilibrium point of (2) is $x_{h+1} = x_h = 0$. We show that, owing to the assumptions made on (2), the scalar function (4) is positive definite whatever the sign of β. Assume that v is semidefinite and denote by N the set on which $v(x) = 0$. Then also $\Delta v(x) = 0$ on N and N is an invariant set of (2), which is possible if and only if N is the point $x = 0$.

Assume that v is indefinite. The integral $\int_0^\sigma \varphi(s)ds$ has its maximum for the linear system $\varphi(\sigma) = k\sigma$ which is, by assumption, asymptotically stable, hence v must be positive definite.[7]

The result proved in this Theorem contains as particular cases those of Szegö and Kalman,[5] and of Zypkin.[8]

3. Consider now some critical cases. Assume that one of the eigenvalues, say γ_k, of the matrix A is unity and all the other eigenvalues of A satisfy (3). Thus, the condition

$$\gamma_i\gamma_j \neq 1 \quad i,j = 1,2, \ldots, n \tag{23}$$

is still satisfied.

Assume that (17), (20), and (21) hold. The Lemma is still valid. Then, by going through the same procedures as in the previous cases, since, owing to (23), the first equation of (13) still defines $H = H'$,[7] one is still able to construct a scalar function of type (4) such that Δv is negative definite along the solutions of the system (2). By the same arguments as in the previous section, one may then show that v is positive definite.

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RESTORATION OF OXIDATIVE PHOSPHORYLATION AND MORPHOLOGICAL INTEGRITY TO SWOLLEN, UNCOUPLED RAT LIVER MITOCHONDRIA

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Recently, in a preliminary report, we described the ability of serum albumin to restore the capacity for oxidative phosphorylation to rat liver mitochondria uncoupled by various substituted phenols.[1] Serum albumin exerts this restorative effect by actually binding and removing the uncoupling compounds from the mitochondria. We now have examined the morphology of mitochondria in the coupled, uncoupled, and recoupled condition. Furthermore, it was of interest to ascertain if another reported beneficial effect of albumin—promoting the ATP contraction of swollen mitochondria[2]—is accompanied by a corresponding restitutive change in mitochondrial structure.

This communication presents evidence that the morphological changes of mitochondria concomitant with swelling and uncoupling are reversible; for, upon the addition of serum albumin and ATP, swollen and uncoupled mitochondria are restored to a considerable extent to their former metabolic and morphological integrity.

The general experimental plan was as follows: equal aliquots of a mitochondrial suspension were added to a Tris-KCl medium (pH 7.4) contained in 3 flasks. One of the flasks served as a control, while the other two contained 5×10^{-5} *M* pentachlorophenol (PCP). Immediately after the addition of mitochondria, the contents of each flask were mixed by gentle swirling, and samples rapidly poured into 3 ml cuvettes. The absorbancy of the suspensions was determined at 520 mμ in a Beckman DU spectrophotometer. When the absorbancy of the suspensions containing PCP had decreased to approximately half of its initial value, serum albumin and ATP were added rapidly to one of the flasks containing PCP. Samples from all flasks again were transferred quickly to other cuvettes, and the absorbancy was redetermined.

After the contraction of the swollen mitochondria was complete, as shown by the absorbancy values, the contents of all flasks were centrifuged. The sedimented mitochondria now were in three conditions: (*a*) normal, unswollen controls (Preparation I); (*b*) swollen by PCP (Preparation II); and (*c*) swollen by PCP and subsequently contracted by the addition of serum albumin and ATP (Preparation III). Portions of each mitochondrial pellet were fixed in buffered osmium tetroxide, embedded in Epon, and examined in the RCA EMU-3G electron microscope. The remainder of the pellet from each flask was resuspended in 0.25 *M* sucrose. Aliquots of these suspensions were incubated in a standard medium for determining oxidative phosphorylation. The PCP and protein content of the mitochondrial suspensions also were determined. Additional details are given in the legends to the figures and tables.

Contraction of Swollen Mitochondria.—Data are presented in Figure 1 which graphically illustrate the immediate swelling which ensued upon the addition of an

uncoupling concentration of PCP to mitochondria suspended in a Tris-KCl medium. It should be pointed out that in a 0.25 *M* sucrose medium much greater concentrations of PCP (10^{-3} *M*) are required to effect an equivalent degree of swelling.[3] As shown in Figure 1, the addition of both serum albumin and ATP resulted in an immediate and rapid contraction of the PCP-swollen mitochondria. Of all nucleotides tested (ATP, UTP, GTP, ITP, ADP), only ATP was effective. It was found in these large-scale experiments that during the contraction induced by 600 μmoles of

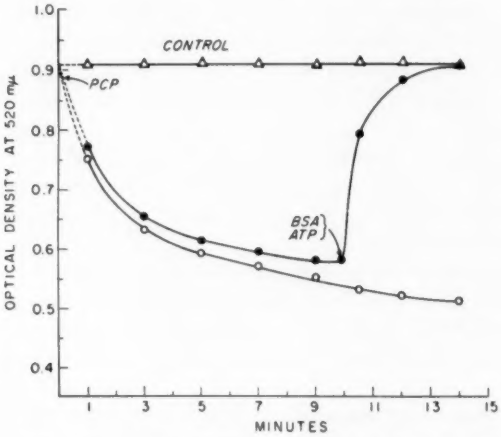

FIG. 1.—Reversal by serum albumin and ATP of the PCP-induced swelling of mitochondria. Four ml of a suspension of rat liver mitochondria (80 mg protein), isolated in 0.25 *M* sucrose (Weinbach, E. C., *Anal. Biochem.*, **2**, 335 (1961) were added to 80 ml of 0.125 *M* KCl–0.02 *M* Tris buffer, pH 7.4, contained in Erlenmeyer flasks. Two flasks contained a final concentration of 5×10^{-5} *M* PCP in addition to the buffer. The volume of all flask contents was adjusted to 100 ml with water. Twice recrystallized bovine serum albumin (BSA) 240 mg, and ATP in a final concentration of 6×10^{-3} *M* were added as concentrated solutions at the time indicated by the arrow. Incubations were at room temperature (24°) for the times indicated. The contents of all flasks then were transferred rapidly to chilled tubes and centrifuged at 10,000 × *g* for 10′ in a refrigerated centrifuge.

ATP, 33 μmoles of P_i were liberated (cf. ref. 4). Dicumarol and antimycin A had no effect on this contraction; oligomycin and gramicidin, in contrast, were distinctly inhibitory. The inhibition by oligomycin was not relieved by 2,4-dinitrophenol.

Independent experiments, summarized in Figure 2, revealed that serum albumin alone, or ATP alone, was ineffectual in promoting rapid reversal of mitochondrial swelling induced by the halophenol. As can be seen in Figure 2, serum albumin alone effected a "passive" contraction[5] of the mitochondria; however, this process was very slow compared to the rapid contraction which ensued upon the subsequent addition of ATP (note time scales of Figs. 1 and 2). Moreover, this passive contraction was insensitive to those inhibitors which prevented the ATP-induced contraction. The addition of Mg^{++} had no detectable effect either on the passive con-

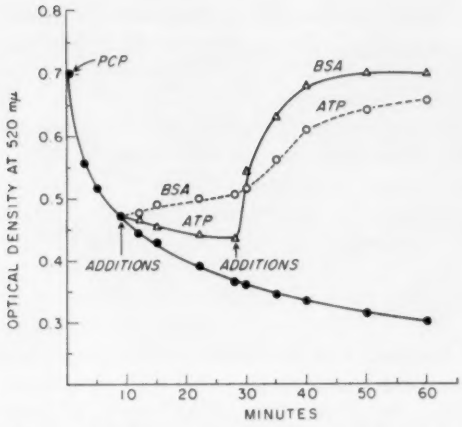

FIG. 2.—Requirement for both serum albumin and ATP for the reversal of PCP-induced swelling. One-tenth ml of freshly isolated rat liver mitochondria was added to 0.125 *M* KCl–0.02 *M* Tris buffer, pH 7.4 contained in cuvettes. Two of the cuvettes contained a final concentration of 5×10^{-5} *M* PCP in addition to the buffer. Final volume was 3 ml. At the times indicated (arrows), 6 mg bovine serum albumin (BSA) or 5×10^{-3} *M* ATP were added as concentrated solutions to minimize dilution effects.

traction promoted by serum albumin or on the subsequent rapid contraction induced by ATP.

Restoration of Oxidative Phosphorylation.—The data in Table 1 show clearly that mitochondria swollen by PCP were completely uncoupled, and that those contracted by the addition of serum albumin and ATP were largely restored in their capacity for coupled phosphorylation. The inclusion of albumin in the medium used to determine oxidative phosphorylation was beneficial to mitochondria in all three conditions and actually conferred a substantial capacity for phosphorylation to the swollen, completely uncoupled mitochondria. The reason for this salutary effect of serum albumin will be discussed below. It is noteworthy that all preparations retained a considerable capacity for oxidative phosphorylation despite the attritions of a prior incubation, resedimentation, and resuspension.

TABLE 1

RESTORATION OF OXIDATIVE PHOSPHORYLATION

Preparation	Condition of mitochondria	Additions	P_i Uptake (μmoles)	O consumed (μatoms)	P:O
I	Normal, control	none	7.0	3.4	2.1
		BSA	7.9	3.2	2.5
II	Swollen	none	0.0	2.7	0.0
		BSA	3.5	3.5	1.0
III	Swollen, then restored	none	2.5	3.2	0.8
		BSA	6.0	3.3	1.8

Mitochondria, sedimented after the swelling experiments, were resuspended in 0.25 *M* sucrose. One-half ml aliquots, containing 5 mg protein, were used in each phosphorylation test. Oxidative phosphorylation was determined with the techniques and medium previously described (Weinbach, E. C., *J. Biol. Chem.*, **234**, 1580 (1959)). Fluoride was omitted. Six mg bovine serum albumin (BSA) added where indicated. β-Hydroxybutyrate was the substrate, the oxidation of which was measured by the stoichiometric accumulation of acetoacetate (Walker, P. G., *Biochem. J.*, **58**, 699 (1954)). P_i uptake was followed by the procedure of Fiske, C. H., and Y. SubbaRow, *J. Biol. Chem.*, **66**, 375 (1925).

The swollen, uncoupled mitochondria (Preparation II) contained PCP in a concentration of 6 μg/mg protein.[6] Other experiments, to be described in detail elsewhere, revealed that a mitochondrial concentration of PCP as low as 0.5 μg/mg protein resulted in complete uncoupling.[1]

The presence of serum albumin in the phosphorylation media resulted in the complete removal of the halophenol from the mitochondria. (The albumin also may bind and remove endogenous uncoupling agents.) This provides an explanation for the partial recoupling of the swollen mitochondria. Even the swollen, then restored mitochondria (Preparation III), which contained less than an uncoupling concentration of PCP prior to the incubation, benefited from the additional albumin in the phosphorylation medium, presumably by a similar mechanism. The effect of serum albumin on the control mitochondria (Preparation I) may be related solely to the binding and removal of an endogenous uncoupler[7] released by the attrition incident to the experimental procedures. One indication of the magnitude of this attrition was shown by the greater ATPase activity in all three preparations compared to the negligible activity of freshly isolated mitochondria.[8]

A comparison was made of the amount of serum albumin necessary to protect mitochondria from the swelling action of PCP to that required for full protection against the uncoupling of oxidative phosphorylation by this halophenol. Previously, we had demonstrated that serum albumin must be present in an equimolar concentration to that of PCP to afford complete protection (or restoration) of phosphorylation.[1] This stoichiometry clearly does not obtain in the albumin protection against PCP-induced swelling. It is evident from the data of Table

TABLE 2

AMOUNTS OF ALBUMIN REQUIRED TO PREVENT SWELLING AND TO PROTECT AGAINST UNCOUPLING

BSA added (μmoles)*	PCP added (μmoles)	F : 0	Swelling absorbancy after 6′
0.0	0.0	2.9	0.89
0.0	1.0	0.0	0.45
0.05	1.0	0.0	0.70
0.10	1.0	0.0	0.88
0.25	1.0	0.0	0.89
0.50	1.0	0.6	0.89
0.75	1.0	1.8	...
1.00	1.0	2.6	...

* Based on a molecular weight of 69,000. Preparations of bovine serum albumin (BSA), whose lipid content was removed by two independent procedures, gave results similar to those recorded here.
Oxidative phosphorylation was determined as described in the legend of Table 1. Swelling experiments were conducted as described in the legend of Fig. 2.

2 that the amount of serum albumin required to protect mitochondria from the swelling action of PCP is much less than the equimolar amount required for protection against uncoupling. It also was found that less than equimolar quantities of the albumin were sufficient as the adjuvant for the ATP contraction of the PCP-swollen mitochondria.

Restoration of Mitochondrial Morphology.—Examination of the morphology of the three types of mitochondrial preparations (i.e., control; swollen; swollen, then restored) revealed that the swollen mitochondria were markedly altered in structure compared to the control, while the morphology of the restored mitochondria resembled that of the control (Fig. 3).

Examination of electron micrographs of thin sections of the control mitochondrial pellets (Preparation I) disclosed that the mitochondria were spherical in shape and

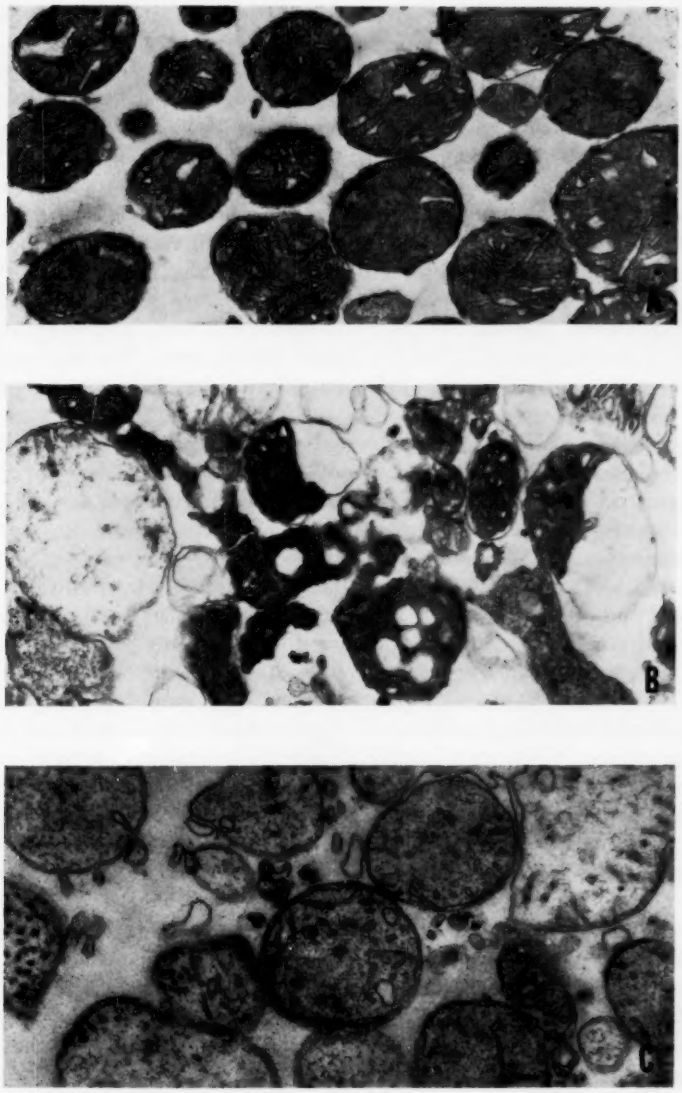

FIG. 3.—Electron micrographs of Preparations I, II, and III. Pellets of mitochondria from these preparations were fixed in buffered OsO_4 (Palade, G. E., *J. Exptl. Med.*, **95**, 285 (1952)), dehydrated in alcohol, embedded in Epon (Sporn, M., T. Wanko, W. Dingman, *J. Cell Biol.* **15**, 109 (1962)), sectioned and stained with lead (Karnovsky, M., *J. Biophys. Biochem. Cytol.*, **11**, 729 (1961)). Magnification *ca.* 25,000 ×. (*A*) Normal, unswollen controls. (*B*) Swollen by PCP. (*C*) Swollen by PCP and subsequently contracted by the addition of serum albumin and ATP.

the cristae slightly swollen. The outer and inner membranes were intact with cristae distributed throughout the matrix (Fig. 3*A*). These preparations compare favorably in morphology with mitochondria fixed immediately after isolation in 0.25 *M* sucrose.

In contrast, mitochondria swollen with PCP (Preparation II) were grossly misshapen. Two distinct types were predominant; one, swollen to several times the size of the control, had a flocculent-appearing matrix which was almost devoid of cristae, and one of the two limiting membranes usually was indistinct or absent. The other type exhibited a condensation of the matrix, some cristae, and a swelling of the outer limiting membrane. The large space between the condensed matrix and the outer limiting membrane was without visible structure (Fig. 3*B*).

The swollen and subsequently contracted mitochondria (Preparation III) had morphological characteristics similar to those of the control preparation. Although these mitochondria were not as spherical or as contracted as the control mitochondria, a matrix entirely filled the space within intact limiting membranes. Cristae were distributed throughout this matrix and resembled those of the controls (Fig. 3*C*).

Discussion and Conclusions.—The capacity for oxidative phosphorylation and the morphology of mitochondria in different states of swelling and contraction have been examined. Specifically, the findings reported in this paper disclose that the biochemical and morphological changes induced in mitochondria by pentachlorophenol are reversible. Serum albumin is both specific and obligatory for the restoration of oxidative phosphorylation; and, except for the standard phosphorylative media,[8] no other adjuvant is necessary. On the other hand, added ATP is needed, as well as serum albumin, for the restitution of normal morphology.

The mechanism by which serum albumin exerts its restitutive effect on oxidative phosphorylation is related to its ability to bind and completely remove the uncoupling phenol from the mitochondria. In contrast, the sequestering of the entire halophenol content of the mitochondria by serum albumin is not required either for protection against swelling (Table 2) or for contraction of the swollen mitochondria. An implication of these findings is that pentachlorophenol induces swelling indirectly, presumably by releasing a potent endogenous swelling agent. A similar mechanism has been postulated for the swelling action of another halophenol, thyroxine.[2, 4] Although characteristics of the swelling induced by pentachlorophenol resemble that induced by thyroxine,[4, 9] there are distinct differences in the response of mitochondria toward these two compounds. The swelling caused by either compound is inhibited by 0.3 *M* sucrose, and its rapid reversal by ATP does not require the addition of Mg^{++}. In contrast, the reversal of the thyroxine-promoted swelling may be accomplished by ATP alone,[4] while serum albumin is obligatory for the ATP-induced contraction of the pentachlorophenol-swollen mitochondria (Fig. 2). Pertinent to these observations is the fact that not all phenolic uncoupling compounds induce swelling of mitochondria. Indeed, 2,4-dinitrophenol not only fails to cause swelling but inhibits the swelling of mitochondria induced by thyroxine and other agents.[9, 10]

Although equimolar amounts of serum albumin are necessary for the protection of mitochondria against the uncoupling action of pentachlorophenol,[1] a much smaller amount of the protein affords complete protection against the swelling induced by

this compound (Table 2). The demonstration that mitochondrial protein is capable of firmly binding pentachlorophenol[11] provides an alternative explanation to that given above for the quantitative aspects of protection by albumin. It has been postulated that ATP induces contraction of mitochondria by interacting with contractile elements in the membranes which presumably are proteins (cf. ref. 10). It is likely that pentachlorophenol interacts with mitochondria at multiple sites, one of which may be a contractile protein. In previous studies we have demonstrated that pentachlorophenol does interact with a contractile protein, myosin.[12] Thus, it is conceivable that serum albumin, in less than equimolar amounts, removes only the pentachlorophenol bound to a contractile protein of mitochondria. Pentachlorophenol so bound may be more readily accessible than that bound to the sites of oxidative phosphorylation.

The restoration of the swollen mitochondria to a morphology resembling that of the controls may be attributed to the contraction of the swollen outer membrane by ATP acting upon an extended contractile protein. This could result in the extrusion of the imbibed water, and the refolding of the condensed inner membrane cristate system (cf. ref. 13). It should be pointed out that ATP generated during oxidative phosphorylation appears to be unable to effect the preservation of normal mitochondrial morphology. For example, in a detailed study to be published elsewhere, we found that mitochondria undergoing active oxidative phosphorylation exhibited marked distortion of their normal morphology. This distortion was evident in mitochondria incubated both in the presence and absence of pentachlorophenol and was little affected by the addition of serum albumin.

Although the swollen, then contracted mitochondria (Preparation III) were largely restored both in their morphological integrity (Fig. 3*C*) and in their capacity for oxidative phosphorylation (Table 1), it should be emphasized that the restitution to normal mitochondrial structure is not a prerequisite for the restoration of coupled phosphorylation. Conversely, it has been shown that the contractile process is retained in mitochondria which have irreversibly lost their capacity for oxidative phosphorylation.[4]

The pentachlorophenol-swollen mitochondria (Preparation II) exhibited a substantial capacity for coupled phosphorylation in the presence of serum albumin (Table 1), despite their markedly distorted morphology (Fig. 3*B*). This is in accord with numerous observations that submitochondrial particles, composed primarily of membrane fragments, can catalyze the coupled reactions of oxidative phosphorylation.[14]

Regardless of the obvious difficulties in the correlation of mitochondrial structure and function,[15] the visible transition from the disrupted, disorganized state of the swollen mitochondria to that of relative structural order seems to us both remarkable and noteworthy.

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*THE INOSINIC ACID PYROPHOSPHORYLASE ACTIVITY OF MOUSE FIBROBLASTS PARTIALLY RESISTANT TO 8-AZAGUANINE**

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Resistance to the analogue 8-azaguanine has generally been found to correlate with loss of guanylic acid-inosinic acid (IMP) pyrophosphorylase activity in several biological systems[1] including 3 mammalian cell lines.[2–4] From 2 of these lines mutants selected for even a small degree of resistance contained very little or no pyrophosphorylase activity.[2, 4] From the other cell line a partially resistant mutant was also isolated in which no change in pyrophosphorylase activity occurred, as well as another mutant (resistant to 8-azaguanosine) which "displayed about one-half of this activity."[3] To investigate further the mechanism of partial resistance we have now isolated several lines of mouse fibroblasts (L cells) which show a small degree of resistance to 8-azaguanine, and have found them to contain 25–44 per cent of the IMP pyrophosphorylase activity of wild-type L cells.

Experimental.—Materials: 8-Azaguanine was obtained from the Francis Earle Laboratories. It was homogeneous on chromatography in isopropanol: H_2O: concentrated HCl (657:176:167).[5] Hypoxanthine-8-C^{14} and 5-phosphorylribose 1-pyrophosphate were obtained from the California Corporation for Biochemical Research, and aminopterin (4-aminopteroylglutamic acid) from the Lederle Laboratories.

Culture methods: An inoculum of L cells was kindly supplied by Dr. L. Siminovitch in 1960, at which time the chromosome number was reported to be approximately 60. This cell line, designated "wild-type," has been maintained in suspension culture in Eagle's "spinner" medium plus 10% calf serum as previously described.[6] It is free of pleuropneumonia-like organisms. Clonal lines were obtained by isolating individual colonies[7] and have been stored at −70°C in medium containing 10% glycerol when not in use.[8]

Determinations of drug resistance or mutant frequency were performed by inoculating an appropriate number of cells (2×10^2 to 5×10^6) from a suspension culture into replicate 60 mm

plastic Petri dishes containing spinner medium supplemented with 10% calf serum, 0.013 *M* $NaHCO_3$, and 0.001 *M* sodium pyruvate, plus the desired concentration of 8-azaguanine. The cultures were equilibrated with 5% CO_2 in air and incubated for 10–20 days at 37°C. When the first grossly visible colonies were noted, the cultures were fixed and stained, and the colonies enumerated (Bactronic Colony Counter, New Brunswick Scientific Co.). For each determination, 3–6 replicate cultures were used, and the colonies were found to be randomly distributed by the chi square test.[9]

Two features of this method deserve mention. To maintain the 8-azaguanine concentration, it was necessary to feed cultures containing more than 10,000 cells, and this was done on the second day and twice thereafter at 2–3-day intervals. Further feeding did not affect the final number of colonies. Without feeding, too numerous colonies or confluent growth occurred, presumably because of incorporation or catabolism of 8-azaguanine, or release of competing purines by disintegrating cells.

The plating efficiency of the cell lines described below ranged from about 10% (AG 3) to 40% (wild-type). However, 2–4 times higher values occurred in reconstruction experiments in which these cells were plated in the presence of 10^6 drug-inhibited cells. Because of this uncertainty, the mutant frequencies below are calculated on the basis of the number of cells inoculated without correction for plating efficiency.

Preparation of cell extracts: About 1.6×10^7 cells were collected by centrifugation, washed with 0.14 *M* NaCl solution at 4°C, suspended in 2 ml of 0.05 *M* phosphate buffer at 4°C and pH 7.5, and disrupted by 3 cycles of rapid freezing and thawing. The resulting suspension was centrifuged at $105{,}000 \times g$ at 4°C for 1 hr. The supernatant fluid was collected, analyzed for protein content,[10] and stored at −70°C. Under these conditions IMP pyrophosphorylase activity was stable for at least 2 months.

Assay of IMP pyrophosphorylase:[11] The incubation mixture (total volume 45 μl) contained about 3 μg of protein; 110 μg of crystalline bovine albumin; 8 mμm of hypoxanthine-8-C^{14} (7.6 μc per μm); 50 mμm of 5-phosphorylribose 1-pyrophosphate; 270 mμm of $MgCl_2$; and 180 mμm of tris(hydroxymethyl)aminomethane at pH 7.4. After incubation at room temperature for 2 hr, 30 μl of the incubation mixture were transferred to ion exchange paper (Reeve Angel paper, grade SB2, loaded with Amberlite IRA-400 resin-Cl^- form). One-tenth *N* formic acid was allowed to ascend through the origin spot, the paper was dried, and the origin spot was cut out, mounted (Tracerlab, Inc.; E-7B rings and disks), and assayed for radioactivity (Nuclear-Chicago model D47 thin end-window counter) to within 5% error. Under these conditions, phosphorylated derivatives of hypoxanthine-8-C^{14} are held at the origin and are present in an amount proportional to enzyme concentration. The reaction was linear for at least 2 hr. An increased concentration of hypoxanthine-8-C^{14} or 5-phosphorylribose 1-pyrophosphate did not increase the rate of the reaction. Essentially no radioactivity was bound at the origin spot when 5-phosphorylribose 1-pyrophosphate was omitted. The specific activity of IMP pyrophosphorylase referred to below represents the counts per minute of phosphorylated derivatives of hypoxanthine-8-C^{14} per μg of cell extract protein per 2 hr at room temperature.

Results.—Clones resistant to 3–4 μg of 8-azaguanine per ml: When wild-type L cells were incubated in the presence of 3–4 μg of 8-azaguanine per ml, the frequency of resistant cells averaged 5×10^{-7} in 6 determinations over a 2-year period (2, 3, 5, 5, 5, and 8×10^{-7}). That such cells were persistently resistant was suggested by the isolation consecutively of 7 colonies, all of which were again resistant to 4 μg of 8-azaguanine per ml after approximately 20 generations in the absence of the analogue. One such colony (AG 3) was recloned and has been grown for over 2 years in the absence of 8-azaguanine, without losing its resistance. In Figure 1 is shown the plating efficiency of wild-type cells and AG 3 cells at increasing concentrations of 8-azaguanine. The AG 3 cells were about 100 times more resistant than the wild-type cells.

It has been shown previously that resistance to 8-azaguanine generally correlates with a marked decrease or absence of IMP pyrophosphorylase activity,[1–4] and

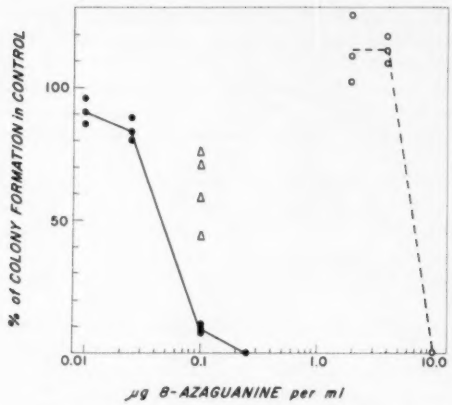

FIG. 1.—Plating efficiency of wild-type (●), AG 46-3 (△), and AG 3 (○) cells at various concentrations of 8-azaguanine (see *Experimental*).

there was no detectable activity in soluble extracts of AG 3 cells (Table 1). Presumably for this reason, AG 3 cells were unable to utilize exogenous hypoxanthine. Thus, there was no incorporation of hypoxanthine-8-C^{14} into the nucleic acids of intact AG 3 cells during 1 hr at 37°C in 2 experiments during which wild-type cells incorporated 370 and 620 cpm.[12]

Clones isolated at a lower concentration of 8-azaguanine: In order to study intermediate degrees of resistance, wild-type cells were incubated in Petri dishes with 0.3 and 1.0 μg of 8-azaguanine per ml. These cultures were not fed, and in retrospect it is unlikely that the level of 0.3 μg per ml was maintained (see *Experimental*). However, several surviving colonies were isolated and enlarged from both the 0.3 and 1.0 μg per ml cultures.

In Table 1 are shown the specific activities of IMP pyrophosphorylase in all the cell lines isolated from the 0.3 and 1.0 μg per ml cultures. The activities fell roughly into 3 groups. Two cell lines (AG 55 and AG 63) selected at 1.0 μg per ml, and 1 (AG 43) selected at 0.3 μg per ml., contained very little activity, resembling AG 3 in this respect. Three lines, (AG 19, 23, and 24), selected at 0.3 μg per ml, contained activity similar to that in wild-type cells. The 2 remaining lines selected at 0.3 μg per ml (AG 41 and AG 46) contained 44 and 35 per cent of the activity of wild-type cells. When examined, the specific activities of these lines have remained approximately constant in a number of preparations during several weeks to months of growth in the absence of the analogue.

TABLE 1

SPECIFIC ACTIVITIES OF IMP PYROPHOSPHORYLASE IN EXTRACTS OF CELL LINES

Cell line	8-Azaguanine* μg/ml	Specific Activity† Low	Intermediate	High
AG 3	2.0	0, 1, 0		
AG 55	1.0	6, 0, 6		
AG 63	1.0	1, 0, 0		
AG 80M	1.0	3		
AG 43	0.3	14, 4		
AG 41	0.3		126, 173, 147, 132	
AG 46	0.3		100, 130	
AG 19	0.3			329
AG 23	0.3			369
AG 24	0.3			239, 260
AG 70M	0.1		98, 99, 123	
AG 55R	—		82	
AG 63R	—		124	
Wild-type	—			369, 320, 291

* Represents the concentration of 8-azaguanine at which the cell line was selected.
† Represents cpm of phosphorylated derivatives of hypoxanthine-8-C^{14} per μg of cell extract protein (see *Experimental*). Each value is the average of duplicate determinations on different cell extracts obtained weeks to months apart.

It seems likely that the IMP pyrophosphorylase activities *per cell* would also fall into 3 groups, as do the activities per μg of protein shown in Table 1. For most of the cell lines, the average amount of protein extracted by freezing and thawing was 97 $\mu\mu$g per cell, with a random distribution of values around the mean ($0.067 < P_{n=9} < 0.091$). For wild-type cells this represented about 40 per cent of the total cell protein. A few extracts were obtained from cells growing slowly in the presence of a partially inhibitory concentration of 8-azaguanine; the average amount of protein extracted was 139 $\mu\mu$g per cell, and, when examined, the cells appeared larger than usual. The specific activity of pyrophosphorylase was similar to that found during more rapid growth in the absence of 8-azaguanine.

Extracts of wild-type, AG 3, AG 43, AG 46, and AG 55 cells were assayed alone and in combination with one another (Table 2). Since in all cases the combined activity was approximately additive, there was no evidence for the presence of an inhibitor or activator in these preparations.

TABLE 2

IMP PYROPHOSPHORYLASE ACTIVITY OF COMBINATIONS OF CELL EXTRACTS

Expt.	Cell extract(s)	Cpm*
1	Wild-type	447
	AG 3	0
	AG 43	14
	Wild-type and AG 3	426
	Wild-type and AG 43	495
2	Wild-type	483
	AG 3	0
	AG 46	186
	AG 55	1
	Wild type and AG 46	675
	Wild-type and AG 55	464
	AG 3 and AG 46	194

* Represents the cpm of hypoxanthine-8-C^{14} converted to phosphorylated derivatives in 2 hr at room temperature (see *Experimental*) by 10 μl of each extract alone or in combination with 10 μl of another extract.

The resistance to 8-azaguanine of AG 55 and AG 63 cells was high and similar to that of AG 3. The resistance of AG 41 and AG 46 cells was slight, but greater than wild-type cells at 0.1 μg of 8-azaguanine per ml (Fig. 1). It was comparable to the first step of resistance to 8-azaguanine in mouse lymphoblasts.[4]

When 5×10^2 AG 41 or AG 46 cells were exposed to a concentration of 8-azaguanine greater than 0.3 μg per ml, no cells survived. Therefore, it was not likely that these cell lines were mixtures of wild-type and highly resistant cells. To exclude this possibility more conclusively, AG 46 was recloned once (AG 46-3) and again (AG 46-3-6 and AG 46-3-7). The pyrophosphorylase activity of AG 46 was 35 per cent, of AG 46-3 was 27 per cent, of AG 46-3-6 was 30 per cent, and of AG 46-3-7 was 39 per cent of the activity of wild-type cells. These results suggested that each cell in the AG 46 culture contained an intermediate pyrophosphorylase activity.

Selection of partially and highly resistant populations of cells: Wild-type cells were incubated in suspension culture with 0.1 μg of 8-azaguanine per ml to determine if this concentration was sufficient to select cells with an intermediate pyrophosphorylase activity. By day 18 resistant cells were apparent (Fig. 2), and these contained 33 per cent of the enzyme activity of wild-type cells (AG 70M, Table 1). That the culture did not consist of a mixture of wild-type and highly resistant cells was indicated by the fact that cell multiplication ceased when the concentration of 8-azaguanine was increased to 1.0 μg per ml on day 30. By day 38, highly resistant cells were apparent, which contained 1 per cent of the enzyme activity of wild-type cells (AG 80M, Table 1).

The generation times of the cells resistant to 0.1 and 1.0 μg of 8-azaguanine per

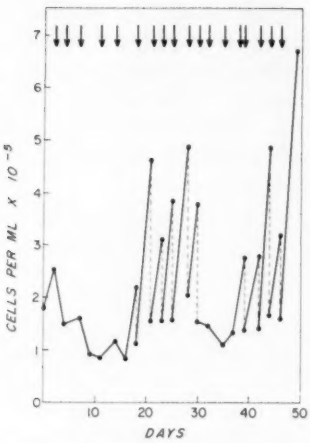

FIG. 2.—Selection of cells partially and highly resistant to 8-azaguanine. Wild-type cells were incubated in suspension culture with 0.1 μg of 8-azaguanine per ml from day 0 to day 30. After day 30 the concentration was increased to 1.0 μg per ml. Feeding with fresh warm medium was carried out regularly (arrows) either by centrifugation and resuspension or by dilution (dashed lines).

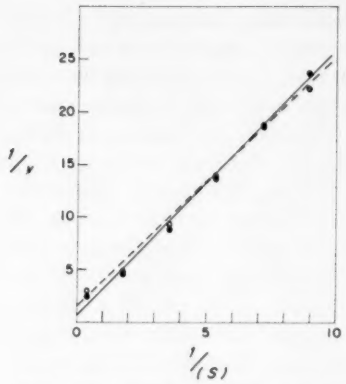

FIG. 3.—Lineweaver-Burk plot to determine the Michaelis constants of IMP pyrophosphorylase in extracts of wild-type and AG 46-3 cells. (S) represents 10^5 times the concentration of hypoxanthine-8-C^{14} in moles/liter, and v represents 10^{-3} times the cpm of hypoxanthine-8-C^{14} converted to phosphorylated derivatives in 2 hr at room temperature by 10 μl of cell extract (see *Experimental*). 5-Phosphorylribose 1-pyrophosphate was not rate-limiting at any concentration of hypoxanthine-8-C^{14} used. For clarity, only the average of triplicate determinations is plotted for each hypoxanthine-8-C^{14} concentration with wild-type (●—●) or AG 46-3 (○---○) cell extracts. The regression lines were calculated from all the determinations by the method of least squares. The standard errors of the estimates of slopes and intercepts indicated no significant difference between the wild-type and AG 46-3 extracts.

ml were approximately 43 and 37 hr (in contrast to 24 hr for wild-type cells). By extrapolation of these values it was estimated that in the inoculum of wild-type cells the frequency of cells resistant to 0.1 μg per ml was about 1.5×10^{-3}, and of cells resistant to 1.0 μg per ml was about 1×10^{-5}. The frequency of highly resistant cells in wild-type cultures was also determined in experiments with Petri dishes mentioned earlier to be about 5×10^{-7}. However, because of low plating efficiency this value doubtless underestimates the true frequency, and may be 2.5–10 times larger (see *Experimental*) and therefore closer to the value of 1×10^{-5} above. In any case these frequencies, while approximate, are consistent with the concept that highly resistant cells arise from cells which are partially resistant.

Properties of the enzyme: To determine if the IMP pyrophosphorylase present in intermediate amounts in AG 46-3 cells was similar to that present in wild-type cells, quadruplicate aliquots of extracts of AG 46-3 and wild-type cells were heated at 87°C for 10 min, and the residual enzymatic activity compared to that in control aliquots. The average drop in activity of AG 46-3 extracts upon heating was 72

TABLE 3

HEAT INACTIVATION OF IMP PYROPHOSPHORYLASE IN CELL EXTRACTS

Expt.	cpm* Wild-type Control	Wild-type Heated	AG 46-3 Control	AG 46-3 Heated	AG 41 Control	AG 41 Heated
1	395	143	308	97	—	—
	448	141	323	98	—	—
	514	163	313	92	—	—
	409	156	350	82	—	—
Mean	442	151	324	92	—	—
% decrease	—	66	—	72	—	—
2	309	122	—	—	261	109
	258	123	—	—	302	99
	376	121	—	—	241	99
	319	127	—	—	251	87
Mean	315	123	—	—	264	99
% decrease	—	61	—	—	—	63

* Represents the cpm of hypoxanthine-8-C^{14} converted to phosphorylated derivatives in 2 hr at room temperature (see *Experimental*). Quadruplicate determinations were done. With AG 46-3 and AG 41 more cell extract protein was used than with the wild-type extract.

per cent and that of wild-type extracts was 66 per cent (Table 3). These results were not significantly different ($0.4 < p_{n=6} < 0.5$). This type of experiment was also carried out with AG 41 (Table 3); in this case the average drop in activity upon heating of the AG 41 extracts was 63 per cent, while that of the wild-type extracts was 61 per cent.

The Michaelis constants of the pyrophosphorylase present in AG 46-3 and that in wild-type cells were determined by the method of Lineweaver and Burk to be 2.9×10^{-4} *M* and 7.4×10^{-4} *M*, respectively (Fig. 3). These values also were not significantly different ($0.2 < p_{n=36} < 0.3$). Together with the heat inactivation studies, they suggest that the enzyme in AG 46-3 cells is the same as that in wild-type cells.

Reversion: In media containing 4×10^{-7} *M* aminopterin supplemented with 3×10^{-6} *M* glycine, 1.6×10^{-5} *M* thymidine, and 1×10^{-4} *M* hypoxanthine, cell growth occurs only if the exogenous hypoxanthine is utilized.[3, 13] Since AG 3 cells are unable to do this, no colonies were formed in this medium by 5×10^{6} AG 3 cells, while the plating efficiency of wild-type cells was not decreased. AG 41 and AG 46 cells survived as well as wild-type cells, and it was not possible to separate these cells from wild-type cells at a low concentration of hypoxanthine.

Under similar conditions, most AG 55 and AG 63 cells, like AG 3 cells, did not survive. However, in both AG 55 and AG 63 cultures, unlike AG 3, the rate of reversion was high, i.e., approximately 10^{-2} and 3×10^{-3}, respectively. It seemed likely that the presence of revertant cells caused the slight pyrophosphorylase activity of AG 55 (Table 1). For both AG 55 and AG 63 a mixture of revertant colonies was grown together over 10 generations in medium containing aminopterin, glycine, thymidine, and hypoxanthine, in order to remove residual highly resistant cells. The pyrophosphorylase activities of these uncloned revertant populations (designated AG 55R and AG 63R) were 25 and 38 per cent, respectively, of the activity of wild-type cells (Table 1). Three clonal derivatives of AG 55R contained 34, 45, and 49 per cent of the activity of wild-type cells. In contrast to these intermediate enzyme levels, Szybalski has described a revertant line of human bone marrow cells which contained "less than one-twentieth" of the IMP pyrophosphorylase activity of wild-type marrow cells.[3]

Attempts to increase enzyme activity: The different IMP pyrophosphorylase activities in these several cell lines could be caused by various degrees of repression of enzyme formation,[14] which aminopterin might be expected to "de-repress." Therefore, pyrophosphorylase activity was determined in several of the cell lines after exposure either to aminopterin, glycine, and thymidine for 16 hr, or to aminopterin, glycine, thymidine, and hypoxanthine for 2–3 days. Under the latter conditions, partially resistant or wild-type cells multiplied almost as well as control untreated cells, whereas the number of AG 3 cells per ml decreased. Essentially no change in pyrophosphorylase activity occurred in any of the cultures under either condition (Table 4). Thus, the enzyme could not be made to appear in AG 3 cells, nor increased in partially resistant or wild-type cells.

TABLE 4

ATTEMPTS TO INCREASE IMP PYROPHOSPHORYLASE ACTIVITY

Cell line	Specific Activity*		
	Control	Aminopterin	Aminopterin and hypoxanthine
AG 3	0	0	0
AG 41	132	—	128
AG 46-3	78	—	69
AG 46-3-6	98	114	—
AG 70M	107	90	—
Wild-type	327	323	352

* See *Experimental.*

Suspension cultures were incubated with 4×10^{-7} *M* aminopterin, 3×10^{-6} *M* glycine, and 1.6×10^{-5} *M* thymidine for 16 hr, or with these 3 compounds and 1×10^{-4} *M* hypoxanthine for 2–3 days. The values for the control specific activities represent one or more preparations.

Discussion.—While genetic studies in tissue culture would preferably be undertaken with diploid cell strains from individuals of known genetic composition,[15, 16] the aneuploid cell lines have such advantages as variety of markers and selection methods, high plating efficiency and mutant frequency, ability to grow in suspension, and recently two putative recombinational techniques.[17, 18]

In the present studies two clonal lines (AG 41 and AG 46) and an uncloned cell population (AG 70M) were selected which showed a small degree of resistance to 8-azaguanine and in which the activity of IMP pyrophosphorylase per μg of protein (and presumably the activity per cell) was reduced to somewhat less than one half of that in wild-type L cells. Pyrophosphorylase activity remained approximately constant in these lines, as well as in wild-type cells, on repeated testing and on attempts to "de-repress" the synthesis of the enzyme. The inactivation upon heating and the Michaelis constants of the enzyme in wild-type and partially resistant cells were not significantly different. These results suggest that such partially resistant cells contain a decreased amount of active enzyme rather than an unchanged amount of enzyme of altered kinetic properties.[19] Finally, the frequencies with which partially and highly resistant cells occurred were consistent with the concept that the partially resistant cell is an intermediate between the wild-type and highly resistant cell.

A possible explanation for these results is that the lines with an intermediate amount of pyrophosphorylase are heterozygous for the ability to synthesize active enzyme, analogous to the situation in individuals heterozygous for galactosemia,[20, 21] acatalasia,[16, 22] and orotic aciduria.[23] If so, the transition from partially to highly resistant cell may involve a recombinational process rather than mutation.[24] The

aneuploid nature of the L cell lines does not preclude heterozygosis, since it is not known if the chromosomes on which the IMP pyrophosphorylase are represented are present in excessive number, nor if so, whether or not they are active. Indeed it is suspected that the supernumerary X-chromosomes present in certain disorders do not function.[25]

It is interesting that an intermediate enzyme activity occurred also in the uncloned revertant populations (as well as clonal derivatives thereof) selected from two highly resistant lines. While this suggests that the revertants too may be heterozygous, the situation is likely to be complex, since several reversion mechanisms, some perhaps extrachromosomal, are known to occur in bacteria.[26]

Summary.—Several lines of mouse fibroblasts with a small degree of resistance to 8-azaguanine were isolated. They contained an amount of inosinic acid pyrophosphorylase activity which was intermediate between wild-type and highly resistant cells. This activity was constant on repeated analyses and despite attempts to "de-repress" the synthesis of the enzyme. The heat inactivation and Michaelis constants of the enzyme were the same in wild-type and partially resistant cells. It is suggested that the partially resistant cells may be heterozygous for the ability to synthesize active inosinic acid pyrophosphorylase.

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EFFECT OF VIRUSES ON EARLY MAMMALIAN DEVELOPMENT, I. ACTION OF MENGO ENCEPHALITIS VIRUS ON MOUSE OVA CULTIVATED IN VITRO

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The possible effect of viruses on mammalian eggs has never been explored. The closest studies that have been reported deal with relatively late embryonic stages.[1] This is unfortunate, since a knowledge of the interaction of mammalian eggs and viruses would be valuable for at least three reasons. First, such knowledge would clarify the known relationship between viruses and congenital defects.[2] Secondly, it would elucidate the origin of innate resistance to virus infection, such as is known in certain strains of mice.[3] Thirdly, it would show us whether the specific cellular receptors required by certain viruses for attachment to the host cell are present on the surface of the vitellus, or whether they are formed later in development.

This communication reports the effect of Mengo encephalitis virus on mouse eggs at the 2-cell stage. The virus was found to pass through the zona pellucida surrounding the egg and to block further development *in vitro*.

Materials and Methods.—Randomly bred 6- to 8-week-old Swiss mice were superovulated by the intraperitoneal injection of 5 I.U. of pregnant mare serum gonadotrophin (Gestyl, Organon), followed 43 hr later by 5 I.U. of human chorionic gonadotrophin (Pregnyl, Organon). A mature male was placed with each female at the time of the second injection. This treatment results in ovulation and mating about 12 hr afterwards. Females with vaginal plugs (usually 70–90% of the animals) were killed 34–36 hr later, that is, 10–12 hr after the expected time of the first cleavage division.[4] The 2-cell eggs were flushed from the Fallopian tubes using a syringe with a blunted no. 30 needle. The medium used for flushing out the eggs and also for culturing them was developed by Dr. R. L. Brinster and is to be published elsewhere.[5] This medium consists of modified Krebs-Ringer balanced salt solution, supplemented with sodium lactate and crystalline bovine plasma albumin.

To determine whether the zona pellucida was a barrier to viral entry it was removed with *Streptomyces griseus* protease ("Pronase," Calbiochem Co.).[6] Ova were exposed 5–10 min at room temperature to 0.25% "Pronase" in phosphate buffered saline, containing 1.0% polyvinylpyrrolidone (PVP). The PVP was added to protect the naked blastomeres and to prevent their attachment to the glass. Further details of the action of "Pronase," and other enzymes, on the zona pellucida of the mouse egg are described elsewhere.[7]

The eggs, either naked or with their zonae intact, were placed in drops of the lactate-albumin medium, with or without virus or antiserum.[7] The drops were submerged in mineral oil in a 60 mm Petri dish to permit gas-exchange while preventing evaporation. The culture dishes were incubated at 37°C in an atmosphere of 5% CO_2 in air.

The 37A (heat-stable mutant) of Mengo encephalitis virus, isolated by Brownstein and Graham,[8] was used. The virus was assayed by the plaque technique decribed by these authors.

Purified virus was prepared by the method of Homma and Graham.[9] In this method nonviral RNA synthesis is blocked in infected L cells with actinomycin D. After lysis the culture is desalted by pouring it through a column of Amberlite mixed-bed ion-exchange resin (MB-1 resin). Zinc hydroxide gel is then added to the column effluent to adsorb and concentrate the virus. Virus is then released from the gel with sodium EDTA and further purified by treatment with trypsin and by two cycles of differential centrifugation. The antiserum used was prepared against the virus of encephalomyocarditis (EMC), which is nearly indistinguishable immunologically from Mengo virus. The rabbit was given two injections of EMC virus grown in L cells. The first consisted of a 100-fold dilution of L-cell lysate and the second of partially purified virus, so that the anti-mouse antibody content was slight. The neutralizing activity of the antiserum was measured at 37° using virus suspensions containing approximately 10^7 PFU/ml. Under these conditions 200-fold diluted serum neutralized 99% of the virus in 10 min.

Results.—Table 1 shows the effect of the virus (L cell lysate) on the development of 2-cell eggs *in vitro*. A variable proportion (22–92%) of eggs with an intact zona developed into blastocysts in the 3-day period of the experiments. Figure 1 shows a group of ova, several of which have developed to the blastocyst stage. These results fully confirm those obtained by Brinster.[5] When the zona pellucida was

TABLE 1

EFFECT OF MENGO ENCEPHALITIS VIRUS* ON THE DEVELOPMENT OF MOUSE OVA *in vitro*

Virus concentration (PFU/ml)	Proportion of 2-Cell Eggs Which Formed Blastocysts in 3 Days					
	Expt. 1		Expt. 2		Expt. 3	
	Zona intact	Zona removed	Zona intact	Zona removed	Zona intact	Zona removed
0	5/23	8/21	12/27	9/25	12/13	6/16
2.8×10^5	...	...	7/24	1/26	10/14	5/15
2.8×10^6	...	...	3/25	0/23	5/15	3/15
2.8×10^7	0/34	0/49	0/46	0/20	0/25	0/13

* Lysate of L strain cells infected with strain 37A (heat-resistant virus mutant).

removed prior to cultivation, blastocysts were still formed. However, in two experiments the proportion doing so was less than when the zona pellucida was present. It was noted that the blastomeres of naked eggs tended to separate at the 4-cell stage. Some of these isolated blastomeres failed to develop further, or formed miniature blastocysts. Occasionally giant blastocysts developed, presumably by the fusion of naked eggs. These effects are shown in Figure 2.

Virus at a concentration of 2.8×10^7 PFU per ml arrested development completely whether the zona was removed from the eggs or left intact. At a concentration of 2.8×10^6 PFU per ml the proportion of eggs which formed blastocysts was reduced, and those eggs which failed to develop became necrotic. At a concentration of 2.8×10^5 PFU per ml the inhibitory and necrotic action was less, but still demonstrable. The effect of Mengo virus on ova with and without their zonae pellucidae is shown in Figures 3 and 4, respectively.

Table 2 shows the results obtained when purified virus was used and the ova observed over a longer period of time. After 3 days the results are the same as those observed with the crude lysate in the previous experiments. However, necrosis of eggs and blastocysts continued with time so that by the 5th day *in vitro* 3×10^5 PFU per ml reduced all the eggs to a necrotic, shriveled condition (Fig. 5). In the absence of virus, blastocysts "hatched" from their zonae pellucidae by the 3rd–4th day *in vitro* and by the 5th day were considerably expanded (Fig. 6). By the 7th day a virus concentration as low as 3×10^3 PFU per ml had the same effect. Since the average drop was about 0.03 ml, this concentration corresponds to 5 PFU per egg.

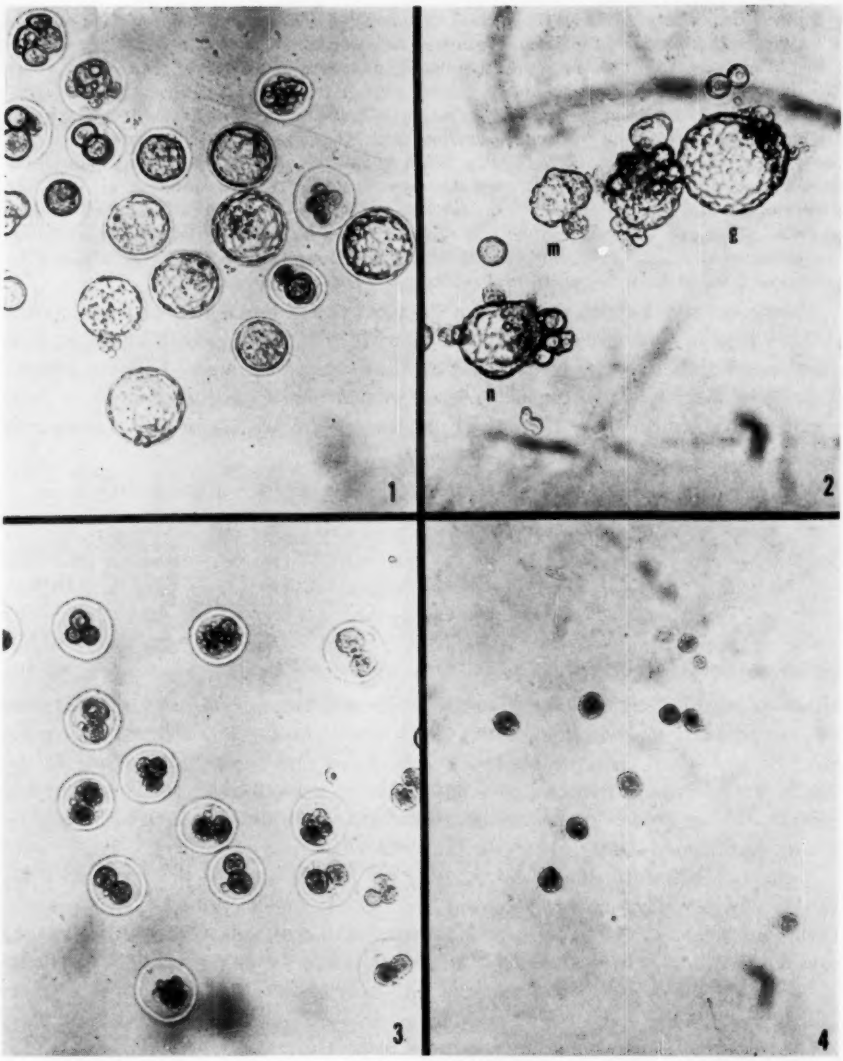

FIG. 1.—Ova, several of which have formed blastocysts after 3 days *in vitro*. × 32.

FIG. 2.—Stages formed from 2-cell ova after removal of the zona pellucida: n, blastocyst of normal size; m, miniature blastocyst; g, giant blastocyst. *In vitro* 3 days. × 32.

FIG. 3.—Ova with intact zonae pellucidae exposed to Mengo encephalitis virus (2.8×10^7 PFU/ml). Further development was blocked and the ova appeared necrotic. *In vitro* 3 days. × 32.

FIG. 4.—Necrotic, shriveled blastomeres from 2-cell eggs without zonae pellucidae, exposed to Mengo encephalitis virus (2.8×10^7 PFU/ml). *In vitro* 3 days. × 32.

The fact that these virus preparations were purified and active at dilutions up to 10^{-6} is strong evidence that the virus itself was responsible for blocking development and producing necrosis of the blastomeres. To confirm this, antiserum was combined with the virus 10 min prior to adding the eggs. Experimental details

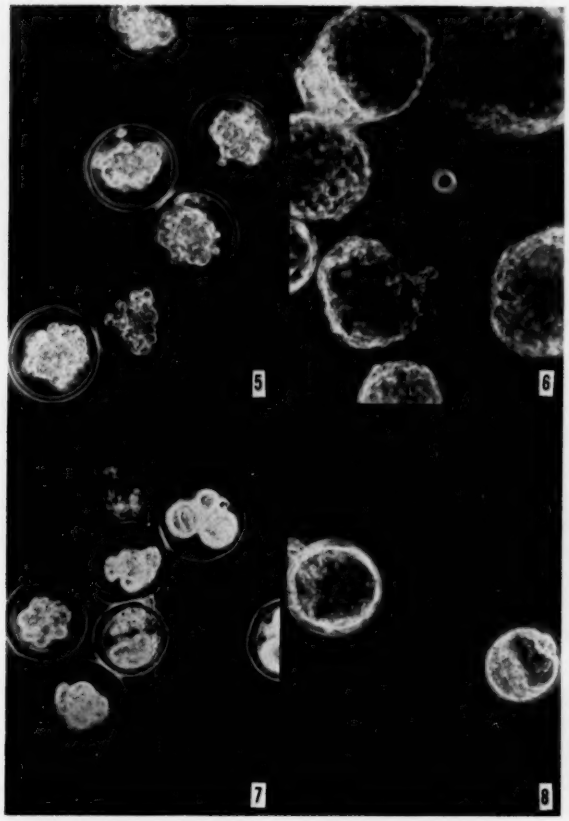

FIG. 5.—Ova exposed at the 2-cell stage to purified Mengo encephalitis virus (3×10^5 PFU/ml). Photograph taken after 5 days *in vitro*. × 50.

FIG. 6.—Blastocysts after 5 days *in vitro*. Hatching from the zona pellucida occurred on the 3rd or 4th day, after which the blastocysts expanded considerably. × 50

FIG. 7.—Ova *in vitro* 3 days after initial exposure to purified Mengo encephalitis virus (3×10^6 PFU/ml). × 50.

FIG. 8.—Blastocysts formed from same batch of ova as shown in Fig. 7, when antiserum was combined with virus. × 50.

TABLE 2

EFFECT OF PURIFIED MENGO ENCEPHALITIS VIRUS* ON THE DEVELOPMENT OF MOUSE OVA† *in vitro*

Virus concentration (PFU/ml)	Days in Culture: 3		5		7	
	Blastocysts	Necrotic cells	Blastocysts	Necrotic cells	Blastocysts	Necrotic cells
0	15	1	17	3	17	3
30	14	0	15	5	15	8
3000	14	3	11	9	0	20
3×10^5	8	8	0	20	0	
3×10^7	0	20	0		0	

* Virus purified by method of Homma and Graham (*J. Cell. Comp. Physiol.*, in press).
† For each treatment 20 eggs were used.

and results are given in Table 3. The antiserum completely prevented the inhibitory action of the virus on development and also prevented necrosis. Figure 7 shows the appearance after 3 days *in vitro* of 2-cell ova exposed to virus. The ova remained undeveloped and became necrotic in appearance. Figure 8 shows blastocysts which formed when antiserum was combined with the virus before adding the ova.

TABLE 3

NEUTRALIZATION OF THE INHIBITORY ACTION OF MENGO ENCEPHALITIS VIRUS ON THE DEVELOPMENT OF MOUSE OVA *in vitro* BY ANTISERUM TO THE VIRUS

Conditions	Number of Morulas and Blastocysts Formed from 15 2-Cell Eggs in 3 Days	
	Morulas	Blastocysts
Medium only	0	9
Virus (3×10^6 PFU/ml*)	0	0
Antiserum (10^{-2})	0	7
Virus and antiserum	1	10

* Purified by method of Homma and Graham (*J. Cell. Comp. Physiol.*, in press).

Discussion.—It is apparent from these results that the zona pellucida is not a complete barrier to virus infection. The only study of zona permeability was made by Austin and Lovelock,[10] who reported that compounds of molecular weight 1,200 or less, but not heparin, which has a molecular weight of 16,000, passed through the zona pellucida of rabbit and rat eggs. Their results suggested that the zona pellucida would act as a barrier to infection of the ovum by viruses. Our results indicate otherwise. However, Mengo encephalitis virus is small, 27–28 mμ,[11] and it will be of interest to determine whether large viruses will also pass through the zona.

While the function of the zona pellucida as a barrier to infection with viruses is in question, our results clearly indicated that the zona was needed to prevent loss of blastomeres or egg fusion under the *in vitro* conditions which were employed. It seems likely that loss of blastomeres and egg fusion could occur within the Fallopian tube if the zona pellucida were not present. Experiments are in progress to establish whether this is in fact true.

The fact that 2-cell ova without their zonae pellucidae developed *in vitro* to the blastocyst stage shows that under these conditions the zona is not required to preserve a microenvironment about the ovum necessary for development. Again, however, whether this is true *in vivo* requires further study. The next phase of this research will be to establish whether Mengo virus is capable of multiplication within the mouse ovum and, if so, whether the yield of virus corresponds to that given by a somatic cell. This problem is being studied by adding virus to ova in microdroplets under mineral oil. After adsorption the virus is washed off and at subsequent intervals the drops are frozen and thawed repeatedly to disrupt the ova and release the virus particles, which are then titrated by a plaque method.

One of the ultimate goals of these studies is to determine in detail the relationship between virus infections and specific congenital defects. The virus spectrum of mouse ova is being determined, and those viruses which do not destroy the ova will be studied further by exposing eggs to virus and then transplanting these ova to the uteri of hormonally prepared foster mothers.

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ERRATA

In the article entitled "Incorporation of Parental DNA into Genetic Recombinants of *E. coli*" by Obaid H. Siddiqi, which appeared in the May issue of volume 49 (1963), the last line of paragraph 3 on page 589 should read "it is S^{32}"; and in the legend to Figure 1 on page 591 "Hfr DNA" should appear as "F^- DNA."

In the article entitled "Synthetic Polynucleotides and the Amino Acid Code, IX" by Albert J. Wahba, Robert S. Miller, Carlos Basilio, Robert S. Gardner, Peter Lengyel, and Joseph F. Speyer, which appeared in the June issue of volume 49 (1963), pages 880–885, *Lys* should be substituted for *Leu* in the amino acids listed in Table 6 under the heading "Triplet composition 2A1U."

In the article entitled "Fluorogenic Substrates for β-D-Galactosidases and Phosphatases Derived from Fluorescein (3,6-Dihydroxyfluoran) and Its Monomethyl Ether" by Boris Rotman, John A. Zderic, and Marvene Edelstein, which appeared on pages 1–6 of volume 50 (1963), the following data should be inserted on page 4 between the two paragraphs of the section entitled "Fluorescein-3-O-methyl-6-(2′,3′,4′,6′-tetra-O-acetyl-β-D-galactopyranoside) (IIg):"

Analysis calculated for $C_{35}H_{32}O_{14}$: C, 62.1; H, 4.8; O, 33.1. Found: C, 62.5; H, 4.8; O, 32.7.

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